

ISOLATION and
IDENTIFICATION



of
*Mycobacterium
tuberculosis*

A GUIDE FOR THE LEVEL II LABORATORY



**ISOLATION AND IDENTIFICATION OF
MYCOBACTERIUM TUBERCULOSIS**

A Guide for the Level II Laboratory

Bobby E. Strong, M.S.
George P. Kubica, Ph.D.

U.S. DEPARTMENT OF HEALTH, AND HUMAN SERVICES
Public Health Service
Centers for Disease Control
Laboratory Improvement Program Office
Laboratory Training and Consultation Division
Atlanta, Georgia 30333

Use of trade names is for identification only and does not constitute endorsement by the Public Health Service or by the U.S. Department of Health, and Human Services.

TABLE OF CONTENTS

	Page
INTRODUCTION TO THE MANUAL	1
References	3
I. SAFETY IN THE LABORATORY	5
A. Introduction	5
B. Personnel	6
C. Room Arrangement and Care	8
D. Recommended Safety Equipment and Supplies	9
E. Precautions in the Isolation Room	15
F. Handling Equipment and Tools Properly	18
References.	34
II. COLLECTION AND TRANSPORTATION OF SPECIMENS	35
A. Collection	35
B. Transportation	39
References.	42
III. DEMONSTRATION AND ISOLATION OF MYCOBACTERIA.	43
A. Introduction	43
B. Isolation Procedures	44
1. N-Acetyl-L-Cysteine-Sodium Hydroxide (NALC-NaOH) Method	44
2. Sodium Hydroxide (NaOH) Method.	51
3. Zephiran-Trisodium Phosphate (Z-TSP) Method	53
4. Cetylpyridinium Chloride-Sodium Chloride (CPC-NaCl) Method	54
5. Oxalic Acid Method	55
6. Sulfuric Acid Method	56
C. Incubation of Cultures	58
References.	60
D. Acid-Fast Microscopy.	61
1. Staining Procedures	63
2. Sodium Hypochlorite (Clorox) Method for Smear Without Culture	70
3. Examination of Smears	71
4. Reporting of Smears	87
References.	88

	E. Culture and Preliminary Observation of Mycobacteria on Lowenstein-Jensen and Middlebrook 7H-10 Media.	89	—
	References.	95	
IV.	SPECIFIC IDENTIFICATION OF <i>MYCOBACTERIUM TUBERCULOSIS</i>	97	—
	A. Speed of Growth	97	
	B. Colony Morphology	98	—
	C. Niacin Tests	101	
	D. Nitrate Reduction Tests	105	
	E. Catalase Test at pH7/68°C.....	110	
	References.	113	—
V.	DRUG SUSCEPTIBILITY TESTS	115	—
	References	123	—
VI.	QUALITY CONTROL PROCEDURES FOR THE LEVEL II MYCOBACTERIOLOGY LABORATORY	127	—
	A. Introduction	127	
	B. General Recommendations	127	—
	C. Laboratory Arrangement and Personnel.	128	
	D. Laboratory Equipment	128	
	E. Glassware	128	—
	F. Media, Reagents, and Biochemical Tests	128	—
	G. Procedure for the Storage of Mycobacterial Stock Cultures	131	
	H. Some Sources of Mycobacterial Stock Cultures	132	—
	References.	132	
VII.	RECORDS AND REPORTS	133	
	Sample Report Form	134	
VIII.	APPENDIX.....	135	
	A. Preparation and Storage of Media.	135	
	1. Preparation of Media.	135	
	2. Storage of Media	138	
	B. Nitrate Reduction Standards	138	
	C. In Case of Accident	140	
	1. Minor Accidents	140	
	2. Major Accidents.	141	
	References.	143	

FIGURES

1. Laboratory suite.	6
2. Isolation room	9
3. Splash-proof containers	13
4. Alcohol-sand flask.	13
5. A transfer spade.	14
6. Equipment in a biological safety cabinet	19
7. Several types of safety pipettors	20
8. Using the rubber-tubing pipettor	20
9. Removal of a screwcap cover from a test tube.	22
10. Inoculating a tube of medium with a pipette .	23
11. Preparing culture suspension from solid medium	26
12. Diagram for tenfold dilutions	28
13. Inoculating plates of media with a capillary pipette	30
14. Preparation of a glass inoculum spreader.	31
15. Inoculating the "spinner" plate	33
16. Packaging and labeling of etiologic agents. ...	41
17. Proper use of test-tube mixer	47
18. Scheme for making tenfold dilutions	48
19. Schematic of a microscope	72
20. Short, long, and clumps of red, carbol fuchsin- stained bacilli on a methylene blue-stained background	75
21. Schematic representation of transmitted-light fluorescence system	77
22. Schematic representation of incident-light fluorescence system	78
23. Schematic representation of blue light bright- field microscopy for acid-fast organisms stained with auramine O.	80
24. Yellow fluorescing, auramine O-stained bacilli. The background fluorescence is quenched with potassium permanganate	81
25. Yellow-orange fluorescing, auramine O- rhodamine B stained bacilli. The background is quenched with potassium permanganate	82
26. A yellow fluorescing, auramine O-stained bacillus on an orange fluorescing, acridine orange-stained background	82
27. Recommended methods for examining acid-fast stained smears.	85
28. Small coccoid forms and branching filaments with beading	86

29. Alternating clear and pink-stained parts of the bacilli that show the characteristic of banding. Note the dark-stained parts called beads	86
30. Strands of bacilli in cords	86
31. Colonies of photochromogenic mycobacteria .	92
32. Colonies of scotochromogenic mycobacteria .	93
33. Colonies of nonphotochromogenic mycobacteria	94
34. Colonies of <i>M. tuberculosis</i> on egg medium ...	99
35. Colonies of <i>M. tuberculosis</i> on 7H-10 agar medium	100
36. Niacin test with chemical reagents.	103
37. Niacin test by the paper strip procedure.	104
38. Nitrate reduction test with chemical reagents .	107
39. Nitrate reduction test by the combined method	109
40. Nitrate reduction test by the paperstrip procedure	110
41. The pH7/68°C catalase test	112
42. Drug susceptibility test medium made with drug-impregnated discs.	118
43. Drug susceptibility test	124
44. Drug susceptibility test	125
45. Drug susceptibility test	126
46. Nitrate reduction standards	139

LIST OF TABLES

1. Levels of laboratory service for mycobacterial diseases	2
2. Preparation of N-acetyl-L-cysteine-sodium hydroxide solution.	45
3. Suggested method for reporting the number of acid-fast bacilli (AFB) observed in smears when scanning at 800X to 1000X	87
4. Preparation of enriched Middlebrook 7H-10 agar from commercially available ingredients	116
5. Drug-containing discs for susceptibility tests. .	117
6. Monitoring of equipment	129
7. Quality control procedures for media, reagents, and biochemical tests.	130

INTRODUCTION TO THE MANUAL



INTRODUCTION TO THE MANUAL

One of the most significant changes in the management of tuberculosis over the past decade has been the trend to treat patients in general hospitals and outpatient clinics rather than within the confines of a sanatorium. This decentralization could result in more and more laboratories receiving fewer and fewer specimens, a change which could cause laboratory personnel to become less proficient in bacteriology at a time when the need for skill in identifying the many mycobacteria now recovered in the clinical laboratory is increasing.

The successful clinical management of tuberculosis has not been paralleled by a commensurate simplification of laboratory procedures or an ease of test performance. To the contrary, many new potentially pathogenic mycobacteria are now recognized, and refined digestion-decontamination procedures are needed to isolate these bacilli from clinical material; requests are made for susceptibility tests against routine and less commonly used drugs and a multiplicity of complex in vitro tests are needed to insure that these new species are precisely identified. Strain-to-strain variation in test response and occasional inherent, erratic test results are some of the often unrecognized problems the clinical mycobacteriologist faces. Unless laboratory personnel perform these in vitro tests frequently, one must question whether they have enough day-to-day experience to maintain equipment, active test reagents, and the expertise needed to properly perform and interpret the many complex procedures required. As a general "rule-of-thumb" it is suggested that proficiency may be maintained by microscopic examination of 10- 15 smears per week, or by routine digestion and culture of 20 clinical specimens per week.

To counter this growing concern about the adequacy of diagnostic laboratory services, the American Thoracic Society issued a policy statement (1) recommending three different levels of laboratory service, the components of which were detailed in a later publication (2). The functions to be performed at each level of service are indicated in Table 1.

The "Levels of Service" program is one of self-evaluation. All laboratory directors take pride in the capabilities of their personnel to perform various procedures and differential tests. Unfortunately, the time may come when a laboratory director finds that certain tests are performed so infrequently that the results are not creditable. The director must then decide either to (1) refer the test or procedure to another institution or (2) upgrade laboratory performance through appropriate programs of training and proficiency testing. This deci-

Table 1. Levels of laboratory service for mycobacterial diseases.

Level	Type of Facility	Type of Service
I	Physician's Office Outpatient Clinic Small Hospitals	Collect good specimens. Ship to Level II or III for culture. Microscopic examination.
	Treatment Center Selected Laboratories (City, County, State, or Private)	Collect specimens. Microscopic examination. Cultural isolation of organism. Identification of <i>M. tuberculosis</i> . Susceptibility tests". Refer "other" mycobacterial isolates to Level III for identification.
III	Reference Laboratory (State, Federal, Private)	All procedures of Level II. Identification of all mycobacteria. Susceptibility tests' *

* Examples of Type of Facility are suggestive, not directive.

** Laboratory personnel should not perform susceptibility tests unless they (1) can identify the organism they are testing, and (2) perform a sufficient number of such tests to be cognizant of the many problems associated with the procedure.

sion is made after a careful review of laboratory procedures and critical self-evaluation. Here are some of the questions that should be answered before the decision is made. What is the frequency of requested service for mycobacteriology in the laboratory? Does the work volume justify the time and effort currently allocated to mycobacteriology? Are you as a laboratory director confident of the results you provide the clinician (are they specific or vague)? Do you have the facilities required for the safe handling of specimens?

No guilt feelings should be associated with a negative response to any of these questions. If bacteriologists would remember that the clinician relies on the laboratory results to establish the diagnosis of mycobacterial disease and perhaps to prescribe curative therapy for his (her) patient, then the importance of accurate, dependable test results, and the need for honest self appraisal of laboratory performance become obvious.

This manual was prepared as a guide for Level II laboratories only. Detailed procedures are presented for the isolation and precise identification of *Mycobacterium tuberculosis*. Methods for rough grouping all other mycobacteria on the basis of pigment production and speed of growth may help the reference laboratories to which

these cultures are referred for more definitive testing. Details for drug susceptibility testing by the submerged disc method are given. A special section on safety emphasizes the importance of individual responsibility in a laboratory, the recognition of potentially dangerous laboratory procedures, and the need for preventive maintenance to insure proper functioning of laboratory equipment.

Detailed step-by-step procedures for all methods are presented, so other publications will only rarely need to be referred to. Selected references are provided at the end of each section. The authors welcome your critical evaluation of this training manual.

REFERENCES

1. American Thoracic Society. 1974. Quality of laboratory services for mycobacterial diseases: Official Statement of the American Thoracic Society. *Amer. Rev. Respir. Dis.* **110:376.**
2. Kubica, G.P., W.M. Gross, J.E. Hawkins, H.M. **Sommers**, A.L. Vestal, and **L.G.** Wayne. 1975. Laboratory services for mycobacterial diseases. *Amer. Rev. Respir. Dis.* **112:773-787.**

**I. SAFETY IN THE
LABORATORY**

I. SAFETY IN THE LABORATORY

A. INTRODUCTION

Laboratory-associated infections are an occupational hazard for all personnel working in institutions where infectious disease agents are handled (7), and *M. tuberculosis* is one of the most important causes of such infections. Studies have shown (3,8) that the risk of tuberculous infection is three to five times greater among workers in the mycobacteriology laboratory than among other workers (secretaries, maintenance personnel, etc.) in the same institution. Most such infections are attributed to the unrecognized production of potentially infectious aerosols containing tubercle bacilli (or other mycobacteria). It is important, therefore, to recognize those procedures which may produce aerosols and to take measures to prevent, reduce, or control them. In the case of *M. tuberculosis*, the most dangerous aerosols are those that produce droplet nuclei (particles $\leq 5\mu\text{m}$ in size). Such particles remain suspended indefinitely in air, and unless some effort is made to remove them, by controlled airflow or ventilation, these droplet nuclei may move freely about the laboratory and may infect people remote from the site where the aerosol originated.

Some laboratory manipulations that have been shown (4,9) to yield aerosols comprised mainly of droplet nuclei $5\mu\text{m}$ or less are:

- Pouring of cultures, supernatant fluids, etc.
- Using fixed volume automatic pipettors
- Mixing a fluid culture with a pipette
- Using high speed blenders
- Using paint shaking machines
- Dropping tubes or flasks of cultures
- Spilling suspensions from pipettes
- Breaking tubes during centrifugation

To minimize the possibility of laboratory-associated infections, we strongly recommend that specially designed suites be used for tuberculosis bacteriology (see Fig. 1). Such sites are of value **only** if they are properly constructed, maintained, and used. Administrators should insure that essential safety equipment is available, and laboratory supervisors should provide necessary training in the handling and use of such equipment. The laboratory worker should be taught how to handle potentially infectious materials in a safe manner, and further instructed that, when handling such materials, he (or she) is responsible not only for his own safety but for that of his co-workers.

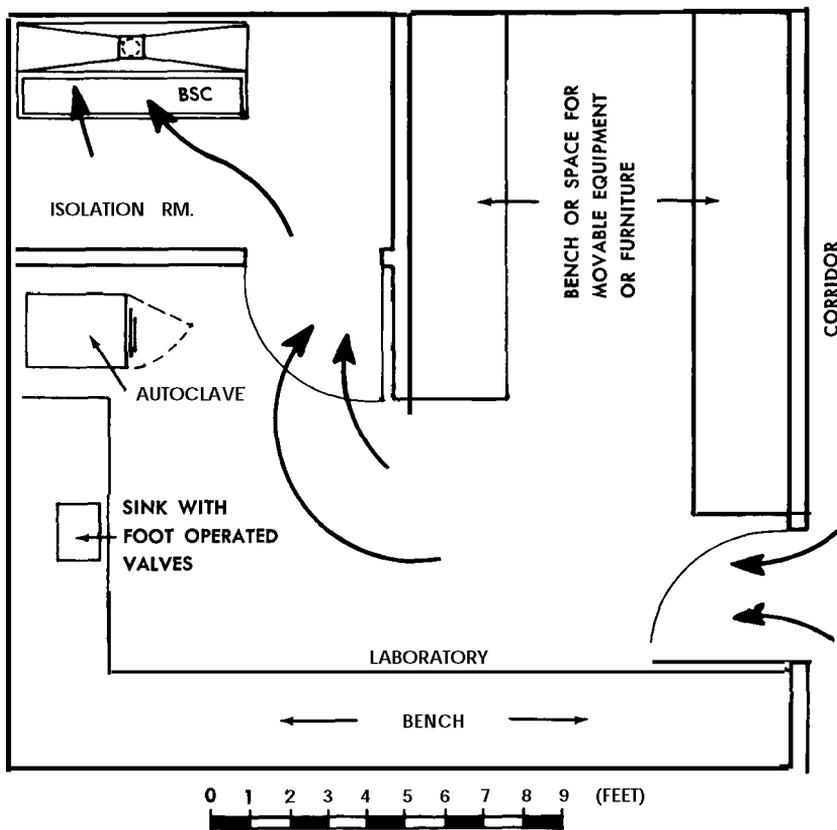


Figure 1. Laboratory suite.

The safety measures recommended in this section were accumulated by personnel during years of infection-free diagnostic and/or research work. Many of the subjects discussed here are detailed in sections on Biosafety Level III in a new CDC publication (12).

B. PERSONNEL

Personnel should be selected with care. They should be physically and mentally capable. Before beginning work in the laboratory, all personnel should have (1) a tuberculin skin test, (2) a chest X-ray, and (3) training in safety techniques and procedures. Other steps in a uniform program of surveillance testing of tuberculosis laboratory personnel are difficult to cite because of many unknown factors. A consideration of two laboratories, the "safe" and the "unknown", may offer guidelines.

1. The Safe Laboratory

A laboratory may be considered safe if all personnel have been trained in correct laboratory procedures, if all equipment (especially the biological safety cabinet) is functioning properly, and if a routine skin testing program has revealed no tuberculin converters among the staff. In such a well-documented situation, tuberculin-negative personnel need not be skin tested more often than annually, and X-rays would be required only if an individual exhibited symptoms. If there is a frequent turnover of personnel (e.g., rotation through the different laboratory services of the institution) skin tests should be done every 3 to 6 months rather than annually.

2. The "Unknown" Laboratory

In this situation little or nothing is known about the tuberculin conversion rate in the staff, i.e., there is no evidence that safety procedures are adequate or that equipment is functioning properly. The following protocol is recommended.

- a. Retest tuberculin negative staff at 4- to 6-month intervals, until it is evident that all equipment is functioning properly and that staff members are adhering to safety procedures (i.e., no conversions are noted). Arbitrarily, 2 to 3 person-years of tuberculin negativity among laboratory workers should be evidence that good safety procedures are being practiced.
- b. Require semiannual X-rays only for tuberculin-positive persons until evidence suggests that this is not necessary (i.e., no converters are seen among the tuberculin negative staff).
- c. Monitor safety equipment and laboratory airflow to insure optimal performance.
- d. Discuss laboratory safety precautions with all laboratory personnel periodically.

If an employee converts from skin test negative to positive, do the following:

Refer the employee to a physician for care or appropriate treatment.
Have the employee check his (or her) laboratory habits.
Reevaluate laboratory safety equipment and procedures.
Remember that even the best safety equipment is no substitute for meticulous technique on the part of a careful, safety-conscious bacteriologist.

C. ROOM ARRANGEMENT AND CARE

Mycobacterial examinations should be permitted only in a laboratory having a biological safety cabinet (BSC) that is tested and certified annually. A safe mycobacteriology laboratory should also have the following features:

1. A self-contained suite housing all equipment needed for processing specimens from receipt to final report (Fig. 1).
2. A well-engineered one-pass ventilation system to maintain airflow in one direction only, from a clean to a less clean area (indicated by bold arrows in Fig. 1). Short strips of tissue paper should be placed on air duct grills in doors and walls and in other appropriate locations as constant indicators of the direction of airflow.
3. An exhaust system discharging air to the outside of the building at the roof or side into an area where the possibility of return to the building is minimal.
4. A separate room, within the suite, designated as the Isolation Room or Contaminated Area (see Figs. 1 and 2) and containing a BSC to be used for all procedures which may generate potentially infectious aerosols (e.g., opening and processing of clinical specimens, preparing slides, inoculating cultures, and performing in vitro tests). The exhaust air leaving the BSC must pass through high efficiency particulate air (HEPA) filters before being discharged to the outside.

Airborne transmission is not the only route of tuberculous infection in the laboratory. The numerous manipulative procedures required to diagnose mycobacterial disease or to identify isolated bacilli, afford many opportunities for accidental infection. Uncovered cuts and abrasions provide a direct route for entrance of surface-contaminating organisms. A microbiologist may accidentally inoculate himself (or herself) with a needle and syringe or he (or she) may puncture the skin with contaminated, broken glassware. Because of these other routes of infection-uncommon though they are-all surfaces and all equipment within the isolation room should be regarded as potentially infectious. These should be regularly cleaned by whatever means is appropriate: disinfectant-swabbing, autoclaving, ultraviolet light. **Good housekeeping is a cardinal rule in the Safe Laboratory.**

General Maintenance in the Mycobacteriology Suite should be performed only under careful supervision. Isolation room equipment should not be serviced, cleaned, or checked unless a trained technical or professional person is present to insure that adequate safety

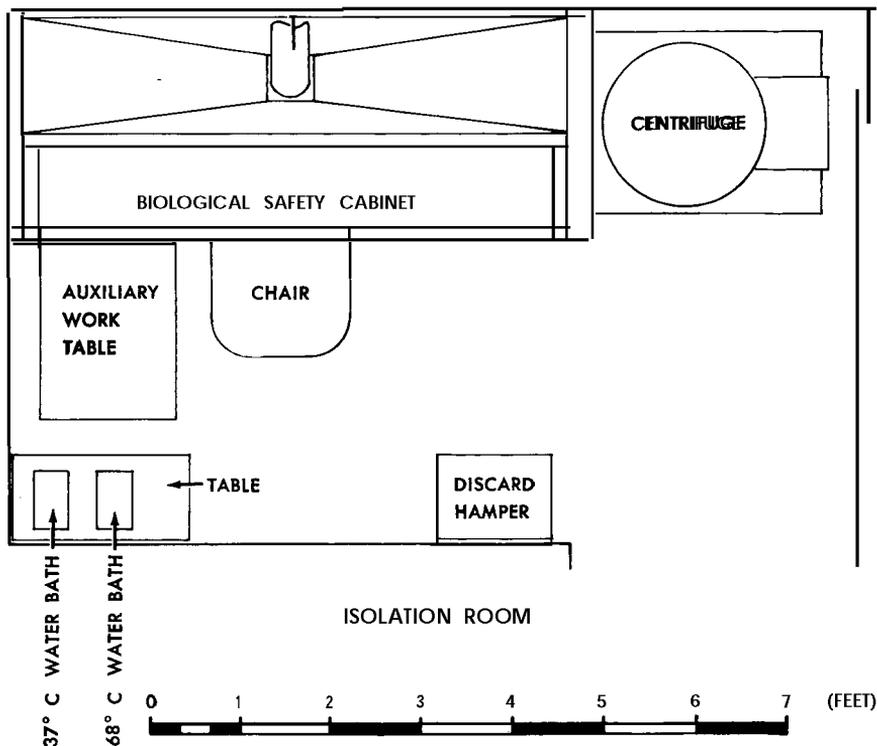


Figure 2. Isolation room.

precautions are observed. Before any major servicing the entire room, including the BSC, should be decontaminated (See VIII. Appendix. C. In Case of Accident, page 140). Later, the room, counters, and equipment should be swabbed with a 3% solution of amphyll (Lehn & Fink Industrial Products Div. of Sterling Drug Inc., Montvale, N. J. 07645) or an equally effective disinfectant. Use rubber gloves.

Unauthorized persons should not be permitted in the Isolation Room. In the event of gross contamination (as by breakage of culture tubes), the entire room must be decontaminated (for details, see VIII. Appendix. C. In Case of Accident, page 140).

D. RECOMMENDED SAFETY EQUIPMENT AND SUPPLIES

Manuals describing the construction, use, maintenance, and decontamination of Biological Safety Cabinets are available (1, 6, 10, 11). These manuals, as well as the descriptive literature from the manufacturer of the safety cabinet(s), should be available to laboratory staff and maintenance personnel.

I. Biological Safety Cabinet (BSC)

The piece of equipment most needed to minimize the possibility of laboratory-associated mycobacterial infection is a well-maintained, properly functioning BSC. This may be either a negative pressure cabinet (Class I) or a vertical laminar flow model (Class II). No laboratory doing diagnostic mycobacteriology should be without a BSC. Use of a BSC does not mean that all chances for laboratory-associated infection are eliminated. Unfortunately, no BSC offers absolute protection from infection. There is still no substitute for care and aseptic technique when performing all laboratory procedures. Detailed instructions for use and maintenance of the Class II laminar flow bio-safety cabinet are presented elsewhere (6, 10, 11). Some basic instructions for properly functioning negative pressure (Class I) BSC follow:

a. Exhaust fan

- (1) Make sure the fan draws a minimum of 75 linear feet of air per minute across the front opening. (The vertical laminar flow, or Class II, safety cabinet is preset at the factory to recirculate 70% to 90% of its air; the 10% to 30% room air drawn through the front opening provides an air barrier to minimize passage of airborne particles into or out from the cabinet work area. Trained or qualified personnel should periodically check the airflow in such cabinets.)
- (2) Operate the fan during all manipulations with specimens, cultures, or animals.
- (3) Check the airflow every 3 months using an anemometer. Magnehelic gauges may be mounted on the cabinet to continuously monitor the pressure drop through the filters (airflow). When the air velocity at the front of a Class I cabinet drops below 75 linear feet per minute, check to see that the exhaust blower fan is operating and that the pulley belts have not been broken. If the fan and pulley belts are functioning properly, it may be necessary to change the air filters in the BSC. Decontaminate the cabinet with an appropriate gaseous formaldehyde (1) and install new filters.

b. Ultraviolet (UV) lights

- (1) If the cabinet is equipped with UV lights for use in decontaminating the work surface, they will be

- mounted within the working area of the cabinet; use bulbs that emit rays primarily of wavelength 253.7 nm.
- (2) Do not operate the UV lights while the cabinet is in use because direct or reflected UV rays may burn the skin or eyes and may kill the mycobacteria.
 - (3) Turn the UV lights on immediately after finishing work in the cabinet and let them stay on for a minimum of 1 to 2 hours. (Continuous operation when the cabinet is not in use costs very little and may be more convenient.) UV light has very little penetrating power and only supplements proper airflow, chemical disinfection, and careful technique.
 - (4) Clean bulbs at least every 2 weeks (more often in excessively dusty areas) with a soft cloth soaked with ethyl alcohol to remove residue and dust.
 - (5) Check UV bulbs at least every 3 months for efficiency of output. Use a portable UV intensity meter capable of measuring UV radiation at a wavelength of 253.7 nm. Visible blue light is NOT a reliable indicator of germicidal UV output (253.7 nm). When output drops below 70% of initial reading for the bulb, replace the bulb.
- c. Place a gauze pad (4-6 thicknesses) or towel (plastic-backed absorbent pads, such as disposable diapers, are acceptable) soaked in disinfectant (e.g., 3% amphyI) over the immediate work area in the BSC. This will minimize spatter or aerosols from spilled fluid, and disinfect viable organisms in such spills.

2. Protective Clothing

- a. Wear a protective outer garment when handling infectious material. The back fastening hospital surgical gown is good because the sleeves fit closely at the wrist and do not drag in the work area. The knitted cuff provides a good "seal" for surgical gloves and protects arms from infectious agents. An added safety factor is that the hospital gown is readily identified as belonging in the isolation room of the laboratory and not in the general work area. Front button laboratory coats are unsuitable (12).

- b. Wear face masks (5) designed to filter >90% of particles in the size range 0.5 to 1.0 μm . Masks must fit the face tightly (e.g., molded or cup-style masks). Remember, no BSC is 100% effective, failures sometimes occur, and the mask adds an additional measure of protection.
- c. Wear rubber gloves; they guard against infection through cuts or abrasions on the skin.
- d. Wear autoclavable or disposable shoe covers and caps; they greatly minimize the chances of removing any infectious agents from the isolation room.
- e. Discard all protective clothing into covered containers or laundry bags and autoclave before washing or disposing of the contents. Clothing, or even glassware, that is tightly packed in a container may have to be autoclaved for up to 2 hours. Use spore strips to determine time required for representative loads.

3. Centrifuges

To minimize chances of infection from aerosols that result when tubes crack or shatter, centrifuge all digested clinical specimens or suspensions of pure cultures in domed O-ring sealed aerosol-free safety carriers. Remove tubes from the safety carriers only inside the BSC (to minimize widespread distribution of aerosol in the event a tube does break in the aerosol-free carrier). An alternative to using safety carriers is to construct a separate air filtration system for centrifuges to control potentially infectious aerosols from shattered tubes (2).

4. Safety pipetting devices

NEVER mouth pipette in the mycobacteriology laboratory. This is not based entirely on fear that infected material may be aspirated, but rather on the fact that a dangerous aerosol results from alternately sucking and expelling fluid through the pipette; in mouth pipetting, the nose is directly over the tube or flask in which the bacterial suspension is being mixed. Several safety pipetting devices are available and should be used. See I.F.2, p. 18.

5. Autoclave

Place the autoclave in a convenient location (preferably within the mycobacteriology suite) so that contaminated material and reusable items can be sterilized before being disposed of or washed. Monitor the autoclave with some sterilization check system.

6. Splash proof container

To minimize the chance of contaminated fluids (e.g., supernatant fluids from digested sputa) splashing back onto surfaces of the BSC, discard them in a special container. Two examples of such containers are shown in Figure 3.

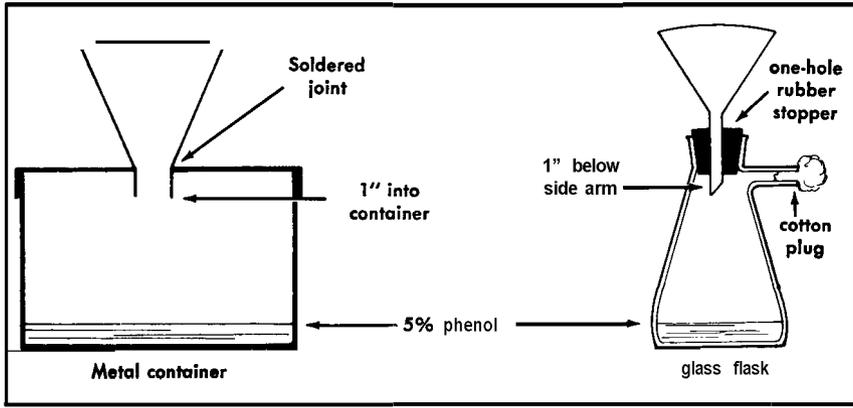


Figure 3. Splash proof containers.

7. Alcohol-sand flask

Use an alcohol-sand flask to remove large clumps of bacilli or organic debris from wire loops or spades before flaming. The abrasive action of the sand cleans most of the debris from the wires and when the loop or spade is placed in the flame of a Bunsen burner, the alcohol causes rapid incineration of residual debris. See Figure 4 below.

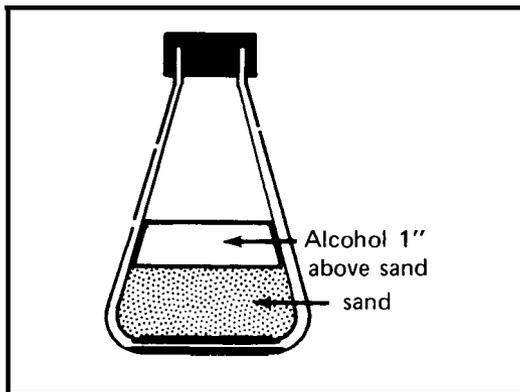


Figure 4. Alcohol sand flask.

8. Culture transfer spade

Use a culture transfer spade to transfer solid culture growth from one medium to another. It is especially useful in making the heavy suspensions recommended for some *in vitro* tests. The spade, made of heavy (18-gauge) nichrome wire flattened on one end by hammering to make an area 5 - 10 mm long by 0.3 to 0.5 mm thick, may be inserted into the handle normally used to hold an inoculating loop or needle. Wire of lighter gauge will bend in the sand. Use the spade with the alcohol-sand flask. See Figure 5 below.

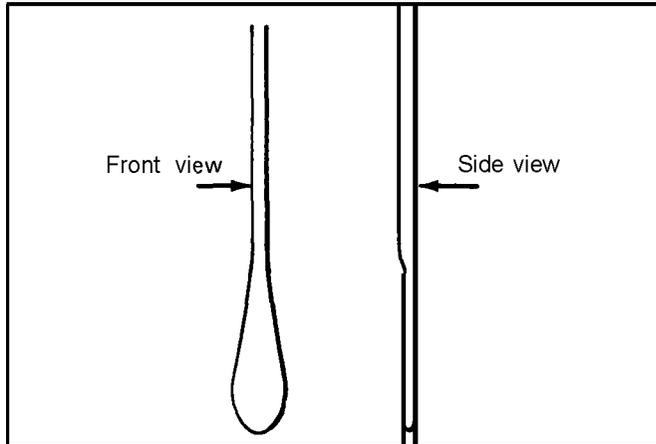


Figure 5. Transfer spade.

9. Other safety equipment and instructions

- a. **BE PREPARED.** Have a container of 3% amphyll in every room where specimens and cultures are handled or stored. In case of an accident, cover broken culture or specimen containers with cloth or paper towels, flood with amphyll, and let stand at least 1 hour before mopping up. If a culture or specimen container breaks in an open room, consider entire area contaminated with aerosol. Consult VIII. Appendix.C. In Case of Accident, page 140.

b. Disinfectants

The killing action of disinfectants or germicides is not instantaneous, but varies from 15 to 30 seconds to 30 minutes or longer, depending upon the concentration, presence of organic debris, and other factors. Some disinfectants, e.g., the quaternary ammonium compounds,

will not kill tubercle bacilli. For general cleanup, use phenolics; use 3% amphy1, 2% cresol, and 3% lysol for tough clean-up jobs in the presence of organic debris, but handle these solutions only with rubber gloves to avoid skin irritation or burns. If contaminated materials are inadvertently handled with the bare hands, rinse hands with 70% ethyl alcohol or a saponated phenolic disinfectant, then wash thoroughly with soap and water.

c. Aerosol-free safety blenders

Although aerosol-free safety blenders permit infected tissues to be homogenized safely, the dangerous aerosol created persists inside the chamber for over an hour, so open only in a BSC. NEVER use ordinary kitchen-type blenders to homogenize infected or possibly infected tissues.

- d. Fit specimen containers and test tubes with screwcaps lined with rubber, teflon, or other plastic.
- e. Have covered discard pans available for all contaminated glassware or material. Be certain disinfectant or water is in the discard pan before the pan (with its contaminated contents) is placed in the autoclave. If the containers must be taken out of the mycobacteriology suite for autoclaving, tape the containers to minimize splatters or aerosols in the corridors. To permit free circulation of steam, remove the tape at the time the container is placed in the autoclave.
- f. If possible, use heat-adjustable slide warming tables set at 65°C to 75°C for heat-fixing smears.
- g. Carry tubes and plates containing cultures to and from the incubators, BSC, and other locations in racks and baskets because they accidentally slip out of bare hands too easily.
- h. If possible, have foot operated controls for soap and water at the lavatories.

E. PRECAUTIONS IN THE ISOLATION ROOM

1. Before entering—

- a. Assemble all materials and equipment. Label specimens, tubes for processing, media to be inoculated, slides, etc., and place them on a laboratory cart to be wheeled into the isolation room.

- b. Have extra supplies (media, dilution tubes, pipettes, and reagents) within easy reach of the isolation room.
 - c. Plan other work to do while in the laboratory, e.g., transfer cultures or make smears. There is often much "dead time" in the isolation room when the centrifuge is running; this time can be used productively if you plan ahead. Keep a check list, if necessary.
 - d. Put on protective clothing listed in D.2, p. 11.
2. **ONCE IN THE ISOLATION ROOM, DO NOT LEAVE UNTIL YOUR TASK IS COMPLETED.** If you need additional equipment or supplies, ask someone to get them for you.
 3. Consider everything in the isolation room potentially infectious. Let no one enter without full protective clothing.
 4. Perform ALL manipulations of specimens and cultures (except centrifugation) inside the BSC (Fig. 6). Work over a gauze pad or towel soaked in 3% amphy. Rewet the pad if it dries out.
 5. Before opening any specimen container, disinfect the outside by wiping it with gauze soaked in 3% amphy. The container may have been contaminated while the specimen was being collected or by leakage in transit.
 6. Autoclave inner metal mailing container before reuse because it may be contaminated.
 7. Place laboratory request slips, which accompany specimens, in a paper bag and autoclave before allowing the record clerk to handle them.
 8. Perform all operations involving agitation of specimens only in aerosol-free containers or within the BSC.
 9. Centrifuge specimens only in aerosol-free safety carriers or in a centrifuge equipped with an air exhaust system (see D.3., p. 12).

10. Heat fix all smears either on a slide warmer at 65°C for at least 2 hours (overnight is acceptable) or by passing 3 times slowly through the blue cone of the flame from a Bunsen burner. If slides are left in the BSC for any length of time, shield them from UV rays which may render some mycobacteria nonacid-fast.
11. BEFORE flaming, clean inoculating loops or spades in an alcohol sand flask (see Fig. 4 and 5).
12. Always use safety pipetting devices (see Fig. 7). DO NOT mouth pipette.
13. Place all materials to be discarded into covered pans containing 3% amphy. Autoclave before sending to washroom. Certain materials may be damaged by autoclaving in phenolics (e.g., reuseable plastic centrifuge tubes may dissolve or craze); in such cases, use water in place of 3% amphy.
14. Discard contaminated or potentially contaminated fluids into splash-proof containers (see Fig. 3) containing disinfectant. Autoclave container before disposing of contents.
15. When work is completed and BEFORE leaving the isolation room:
 - a. Cover all discard pans. Contaminated material must be in covered containers when brought out of the isolation room.
 - b. Wipe the outsides of discard containers and culture tubes with disinfectant soaked gauze before bringing them out of the isolation room. Dip racks of culture tubes into a disinfectant bath (up to the screwcap) instead of swabbing individual tubes.
 - c. Swab the work area of the BSC, counters, and cart with 3% amphy or some other disinfectant. Scrub the area periodically with detergent solution to remove the oily residue of some disinfectants.
 - d. Place the gauze pad which covered the work area in a covered container containing 3% amphy.

- e. Turn on UV lights in the work area of the BSC (before doing so, remember to cover any microscope slide smears that are left in the BSC).
 - f. Remove protective clothing (the MASK LAST) and place in a covered container or laundry bag, which is left inside the isolation room. When the container or bag is full, autoclave it before laundering or disposing of contents.
16. Upon leaving the room, scrub hands and arms well with soap and water. Do not wear watches or bracelets in the BSC room because they interfere with thorough cleansing of hands and arms.

F. HANDLING EQUIPMENT AND TOOLS PROPERLY

BEFORE beginning any work with potentially infectious material, be certain you know how to operate all the safety equipment you have and how to use those laboratory tools which can greatly minimize chances for laboratory-associated infection. Familiarize yourself with these tools. Try them first with a beaker of water or a heat-killed culture.

Work in an uncluttered area. This alone will reduce greatly the possibility of accidents.

1. Arrangement of equipment in the Class I BSC

Figure 6 shows a suggested arrangement of the work area in the BSC. NOTE ESPECIALLY that the area immediately in front of the worker is clear, thereby facilitating the handling of specimens and cultures. Any drops or spatter from pipettes, loops, tubes, etc. will probably fall onto the disinfectant-soaked gauze, rather than onto other tubes or racks in the work area.

2. Safety pipettors

Several kinds are available commercially. Two that have the advantage of handling pipettes of variable volume (if needed) are the Propipettor and the Caulfield pipettor; follow manufacturer's directions for use. Rubber bulbs are sometimes used on pipettes of 1 -ml volumes or less.

An inexpensive pipettor for use with pipettes of 1 -ml volume or less may be made by cutting 2 1/2-to 3 -inch pieces of flexible rubber tubing that will fit snugly over the pipettes you will use and inserting a small cork or rubber stopper in one end of the tubing.

To operate, simply place the rubber tubing on a pipette, and BEFORE inserting it into the bacterial suspension you wish to pipette, expel the air from the pipette by rolling the tubing over the thumb of your right hand (the hand holding the pipette). After inserting the pipette tip into the bacterial suspension, gradually release the “rolled” tubing back to its

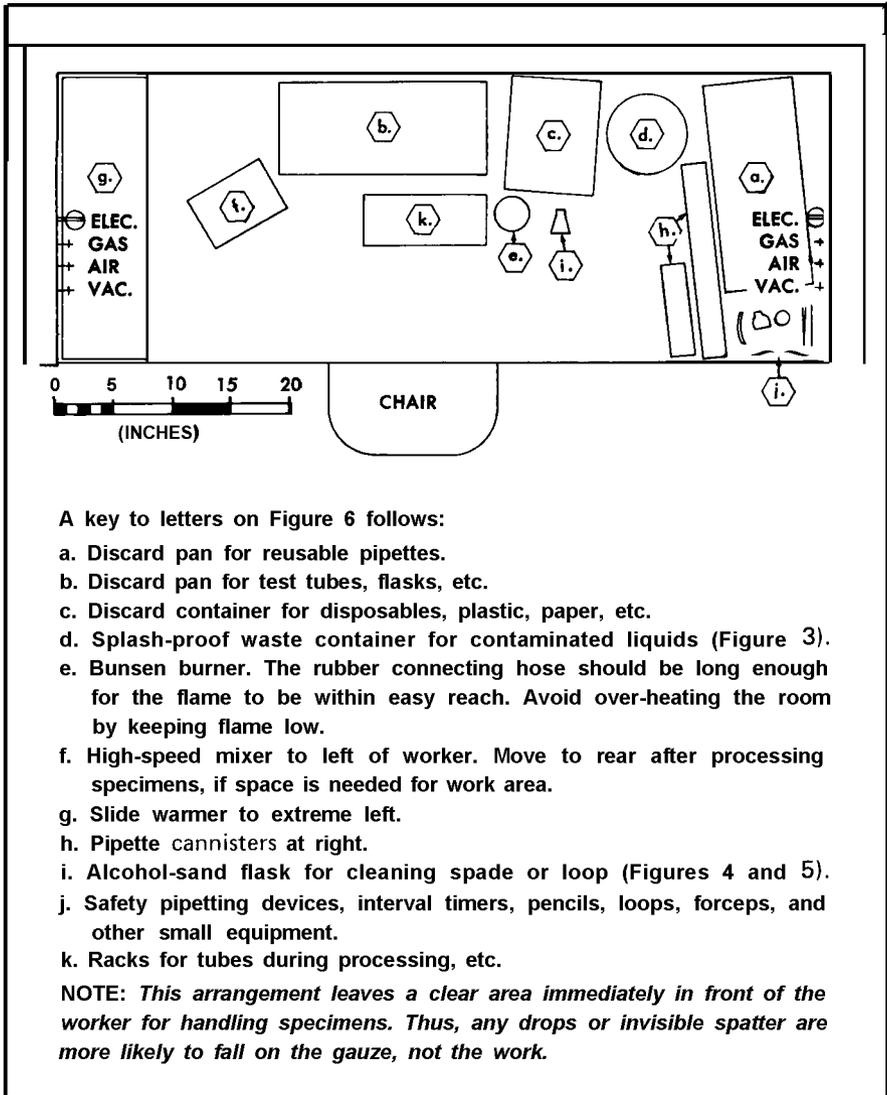


Figure 6. Equipment in a biological safety cabinet (modifications may be necessary for other types of safety cabinets).

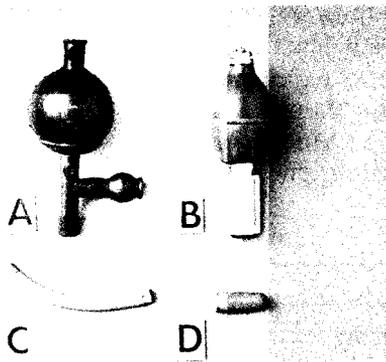


Figure 7. Several types of safety pipettors: (a) Pipette filler; (b) Caulfield; (c) Rubber tubing pipettor (home made); (d) Rubber bulb.

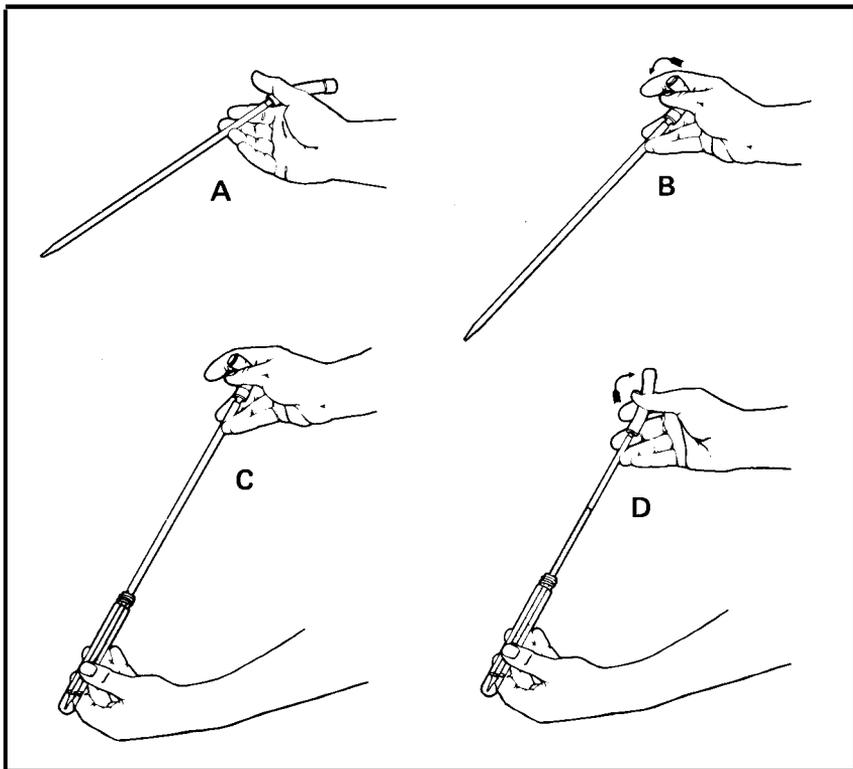


Figure 8. Using the rubber tubing pipettor.
 A. Cradle pipette over index finger and between tips of second and third fingers.
 B. Expel air by "rolling" rubber tube over thumb with index finger.
 C. Insert pipette into fluid suspension.
 D. Suck up inoculum by slowly "unrolling" rubber tubing.

normal position, thereby aspirating the culture suspension into the pipette.

Figure 7 shows several of these pipettors, and Figure 8 shows how to use the “rubber tubing” pipettor.

Practice using one of these types of pipettors with a beaker of tap water until you can control both the aspiration and delivery of desired volumes of fluid.

3. **Proper removal of screwcap cover from test tube**

Because most mycobacteria have a long generation time (12-18 hours), media inoculated with these organisms must sometimes be incubated up to 6 to 8 weeks or longer before being discarded. To retard dehydration during prolonged incubation, place inoculated media in screwcap tubes or bottles (exceptions are the oleic acid albumin agar media commonly placed in Petri dishes).

To minimize contamination of screwcap-covered media, use aseptic technique in removing caps and inoculating media, just as one would use care in removing a cotton plug from a tube of medium in the general bacteriology laboratory.

DO NOT remove caps from tubed media and place them on the work surface while the medium is being inoculated. This is an open invitation to more rapidly growing (12-18 minutes generation time) contaminating organisms, which can ruin your tuberculosis (TB) isolation media.

Assuming you are right-handed, (left-handers will use the opposite hands) grasp the test tube in the left hand. Place the little finger of the right hand around the screwcap (see Fig. 9). Hold the right hand rigid, and with the left hand unscrew the tube from the cap (the reason for this is that you may also be holding a loopful or pipetteful of inoculum in the right hand and you do not want to be waving this around in the air).

The opened tube should be held at about a 45° angle, with the opening facing the inside of the BSC to minimize contamination.

Hold the cap in your little finger while you remove inoculum from or inoculate the medium in the tube.

NEVER set the cap down on the work surface.

To reclose the tube, again hold the right hand rigid, and with the left hand move the test tube up to the cap which is still held by the right little finger. Once the tube is inside the cap, screw it down tightly with the left hand (see Fig. 9).

4. **Inoculating tubes of media with loops, spades, or pipettes.** The procedure for inoculating tubes of media with loops, spades, or pipettes varies only slightly from that described in F.3., above and Figure 9. Remove and replace the cap exactly as before, except hold the tool for inoculating the screw-capped tube of medium in the right hand.

By using only the little finger to remove the test-tube cap, you have the thumb and first three fingers of the right hand to hold a loop, spade, or pipette.

Loops or spades can be easily held with the thumb and first two fingers. Because the pipette with an attached rubber tubing safety pipettor may be the most difficult to maneuver, we will outline the procedure.

Cradle the pipette over the index finger and between the tips of the second and third fingers, as shown in Figure 10.

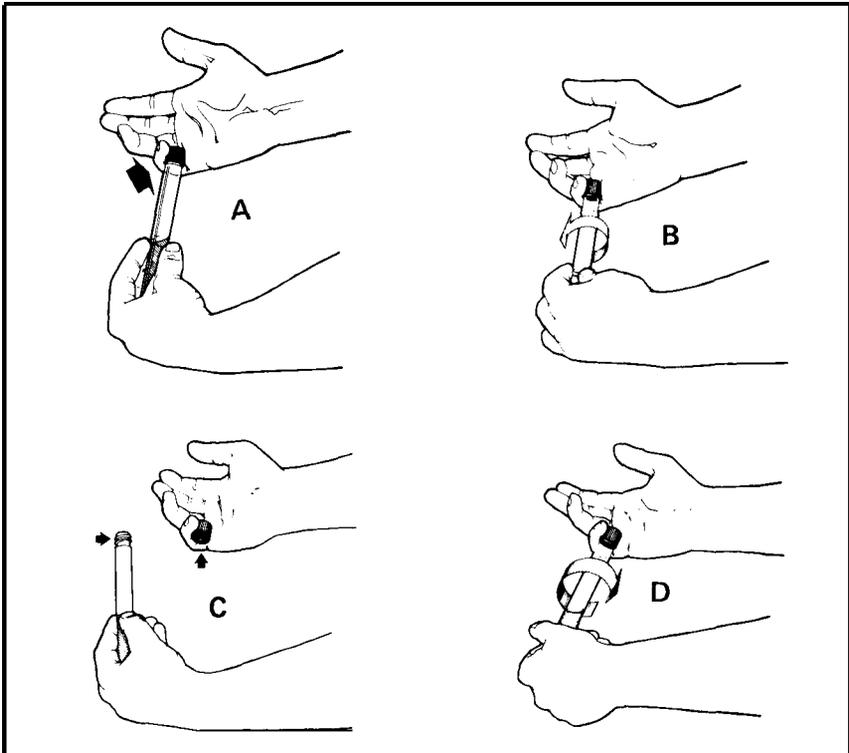


Figure 9. Removal of a screw-cap cover from a test tube.

- A. Place little finger of right hand around screw cap.
- B. Hold right hand rigid and unscrew tube from cap by turning left hand.
- C. Remove tube from cap, but always point open end inward at about 45° angle.
- D. Recap tube by holding right hand (and cap) rigid, while using left hand to screw tube back into cap.

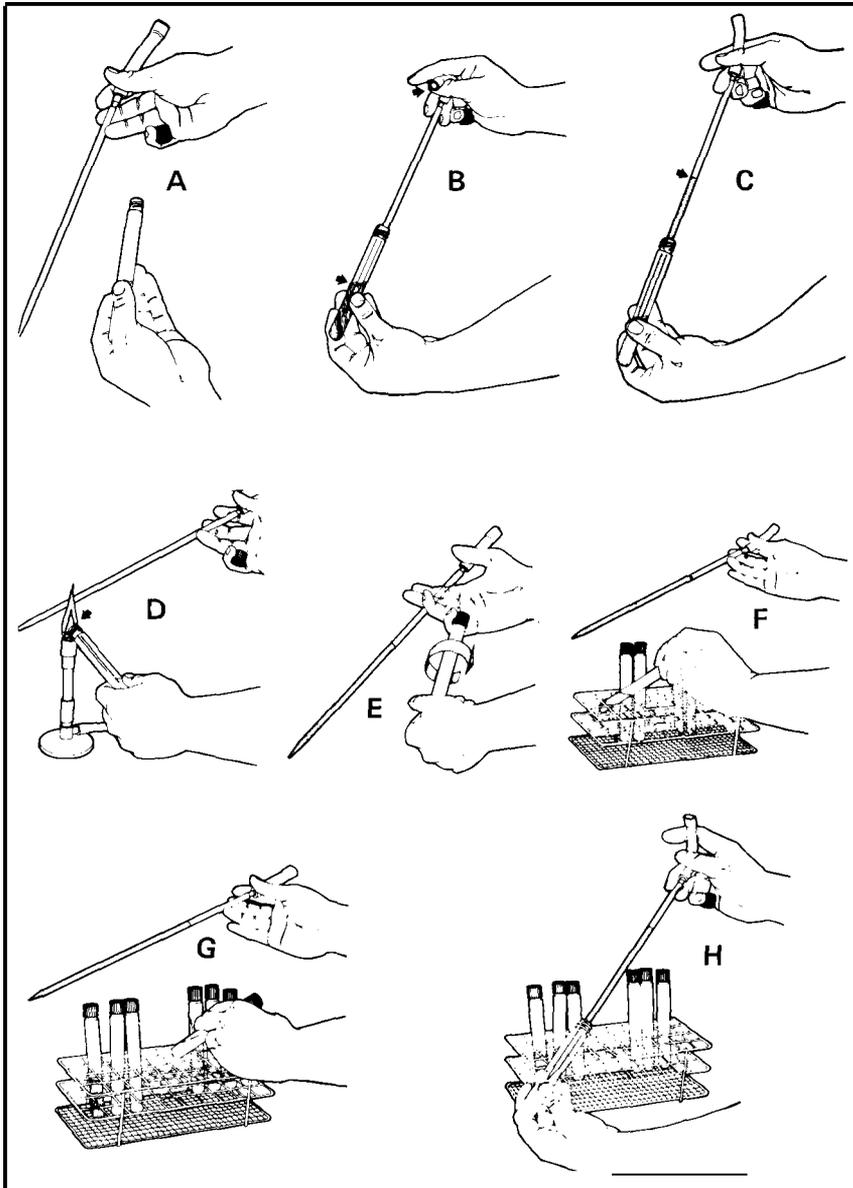


Figure 10. Inoculating a tube of medium with a pipette.

- A. Pipette cradled in rigidly held right hand; screw cap removed *from* tube (see also Figures 8 and 9).
- B. Roll tubing over thumb and insert pipette into microbial suspension.
- C. "Unroll" tubing to suck up inoculum.
- D. Hold pipette hand still; remove tube from pipette and flame tube opening.
- E-F. Recap the tube and *replace in* rack.
- G. Select from rack the tube of medium to be inoculated.
- H. Remove cap in usual way, slide tube over pipette tip, and deposit desired amount of inoculum.

Remove the cap from the test tube, using the little finger of the right hand, as detailed in F.3., p. 2 1.

Hold the pipette hand stationary and unscrew the tube with the left hand.

When the cap is off, expel the air from the pipettor by folding the rubber tube over the thumb with the index finger (see F.2., p. 18, and Figure 8); insert the pipette into the opened tube and suck up the desired inoculum.

Hold the pipette hand still, and REMOVE THE TUBE FROM THE PIPETTE instead of pulling the pipette out of the tube.

While holding the pipette hand rigid, flame* the mouth of the open test tube and move it back into position in its cap. Tighten the tube and replace it in its rack.

Holding the pipette hand still, use the left hand to pick up the tube to be inoculated, and move the cap into position in the little finger of the right hand.

Go through the tube-opening process again.

Tilt the pipette tip downward slightly, to meet the open mouth of the now uncapped test tube. Deposit the desired inoculum, remove the tube from the pipette, flame, and recap as before.

Use the same procedure when carrying the inoculum on a spade or loop.

REMEMBER, keep the hand which holds the inoculating tool as still as possible because any excess movement could release potentially infectious aerosols.

5. Preparing culture suspensions from solid medium

Using the technique described in 3. and 4., pp. 21-22, aseptically add 0.5 ml of sterile water or saline to a sterile screw-cap test tube. Take the wire spade in your right hand (see Figure 1 la) and sterilize it by heating it red hot in the blue cone of the Bunsen burner flame. Let the spade cool.

*Flaming the mouth of the test tube is an optional procedure. Flaming probably is not necessary in laminar flow safety cabinets (Class II). With negative pressure (Class I) cabinets, decide whether to flame after determining the extent of contamination encountered without flaming.

Pick up in your left hand the culture slant from which the bacterial suspension will be made and carefully remove the screwcap as described in 3. (p. 21) (see Figure 1 1b). Now flame the mouth of the tube.

Carefully insert the spade into the tube and position it so that the long flat plane of the spade is perpendicular to the surface of the slanted medium (Fig. 11 c). Scrape some growth off the surface of the culture slant with the spade. CAUTION: You only want bacterial growth, not medium, so avoid scraping up pieces of the medium.

Carefully remove the spadeful of growth from the culture tube, flame the tube, and recap it as in 3., p. 21.

Pick up the tube containing the 0.5 ml sterile water, and carefully unscrew the cap. DON'T FORGET to hold the hand with the spadeful of growth stationary. The left hand does all the moving.

Flame the mouth of the tube and tip it so that the water flows up and wets the wall of the tube about 1 inch (25 mm) above the fluid surface (see Fig. 1 1d).

Bring the mouth of the tube up to the bacteria-laden tip of the wire spade.

Insert the spade into the tube, being careful not to touch the sides of the tube because this might dislodge some of the growth.

Bring the tip of the spade (with bacteria on it) down to the portion of the tube with the wet wall.

Turn the spade so the bacterial growth faces the wet glass wall of the tube (Fig. 1 1e).

Macerate the bacilli by moving the spade in a circle against the wet wall of the tube (Fig. 1 1f).

Always hold the tube at a 45° angle with the open end facing slightly inward to keep airborne contaminants from entering during this sometimes lengthy process.

When maceration is complete, use the tip of the spade to push the bacterial mass into the reservoir of fluid at the bottom of the tube (Fig. 1 1g).

Your inoculum is now ready for further standardization, if necessary, e.g., by turbidimetric methods or comparison with optical density standards.

See Figure 1 1 for details.

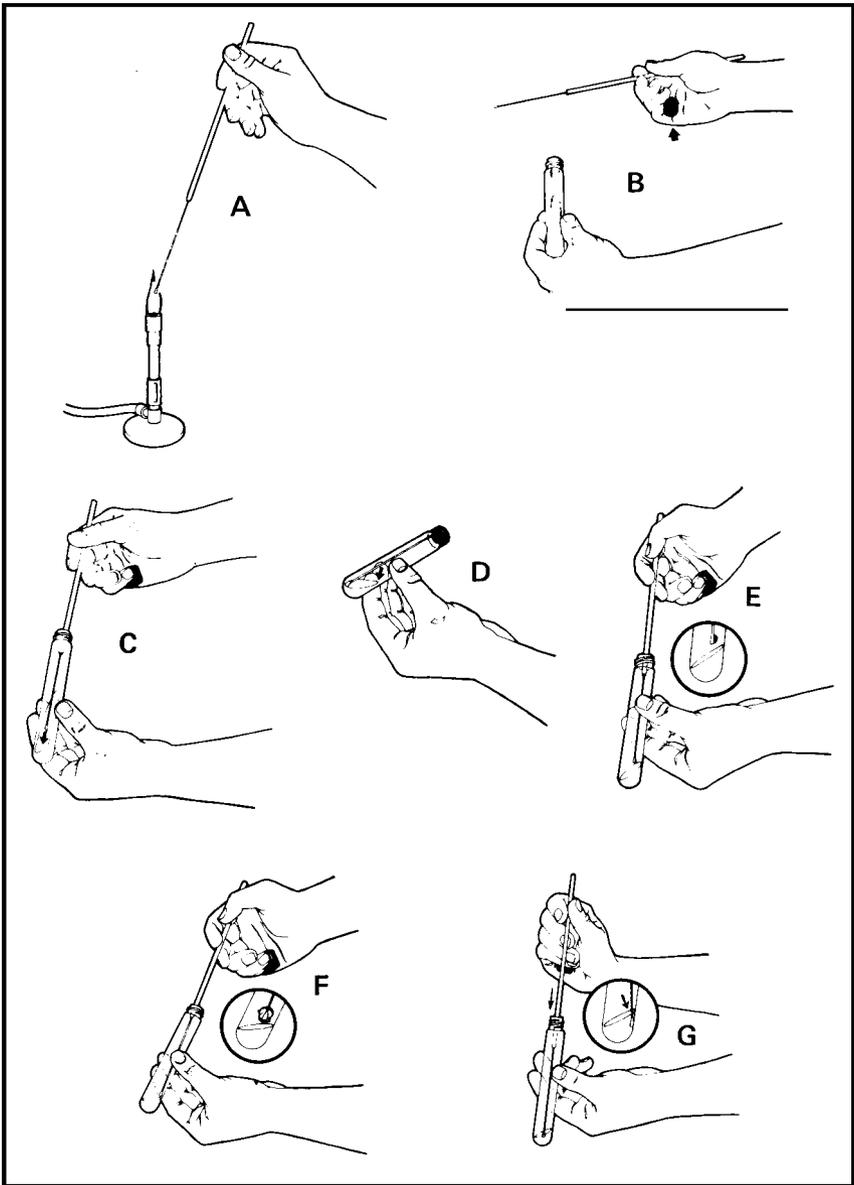


Figure 11. Preparing culture suspension from solid medium.

A. Sterilize inoculating spade in Bunsen burner flame.

B. Remove screw cap from tube of culture. (See Figure 9.)

C. Insert blade of spade so long flat plane of spade is perpendicular to surface of medium; carefully scrape up bacterial growth. Recap culture tube and return to rack.

D. Tip test tube of water so that fluid wets wall 1 inch (25mm) above fluid surface.

E. Remove cap from tube of water and insert bacteria-laden spade and turn bacteria toward the wet wall of test tube.

F. Macerate bacillary mass by moving spade in circular motion against wall of test tube.

G. Push macerated bacilli down into fluid with tip of spade.

6. Preparing serial tenfold dilutions

This requires a combination of the procedures described in 3. and 4. (pp. 21-22).

Serial tenfold dilutions of a heavy bacterial suspension are frequently needed in the mycobacteriology laboratory to (a) obtain isolated colonies on media later inoculated with a suitable dilution, (b) provide proper inoculum size for certain in vitro tests, or (c) prepare a bacterial suspension containing appropriate numbers of bacterial cells to insure a valid drug susceptibility test result.

To obtain these tenfold dilutions, make a series of successive transfers of 1 part of bacterial suspension to 9 parts of sterile, bacteria-free diluent (water or saline). Do this by transferring 1 ml of bacterial suspension to 9 ml of sterile water (or 0.5 ml of bacterial suspension to 4.5 ml of diluent).

Aseptically pipette the dilution blanks (4.5- or 9.0-ml amounts) into sterile tubes as described in 4. p. 22 above by using a Propipettor or other pipettor for larger volumes of fluid. Alternatively, add the desired fluid volumes to non-sterile screwcap tubes, tighten the caps securely, and sterilize the tubes in the autoclave (121 °C/15 minutes).

Place the starting bacterial suspension, with the desired number of dilution blanks, in a test-tube rack. Leave the first hole of the rack empty; then, as each tube is opened and used for dilutions, move it one place to the left in the rack, thereby indicating that inoculum has been removed from that tube and used (this avoids the confusion of not knowing which tubes have been used and which have not).

Prelabel all dilution tubes with the final bacterial dilutions they will contain. The starting suspension is undiluted (U or 10^0). The first tenfold dilution is 1:10 (or 10^{-1}), the second 1:100 (or 10^{-2}) the third 1:1000 (or 10^{-3}), etc.

Premix the starting bacterial suspension on a test tube mixer to get a homogeneous mixture.

Using skills learned in 3. and 4., pp. 21-22, aseptically remove the screwcap from the starting suspension, flame the mouth of the tube, and remove exactly 1.0 ml (or 0.5 ml) of bacterial suspension from the tube, using a pipette and safety pipettor.

Flame the original tube, replace the cap, and place the tube in the left-most hole in the rack (indicating you are finished with the tube). Quickly pick up the " 10^{-1} tube" in your left

hand, aseptically open, and add the contents of your pipette to this tube.

Flame the tube, replace the cap, and then discard the used pipette into a long, low pipette discard pan containing some phenolic disinfectant (see Fig. 6).

Place this newly inoculated "10⁻¹ tube" onto a test-tube mixer to thoroughly mix the contents. The bacterial population in this diluted tube now contains 10 times **fewer** (one tenth as many) bacteria per unit of volume than does a similar volume from the undiluted tube.

Place the safety pipettor onto another sterile pipette, and using procedures in steps 3. and 4. (pp. 21-22), remove the cap from the "10⁻¹ tube," flame the mouth, and remove exactly 1.0 ml (or 0.5 ml) of the suspension from this tube.

Flame the opening of the "10⁻¹ tube," recap it, and place it in the rack immediately next to the undiluted tube.

Pick up the "10⁻² tube" in your left hand, open and flame it, and add to it the contents from the pipette.

Flame the tube, replace the cap, and then discard the used pipette as described above.

After mixing this "10⁻² tube" on a test-tube mixer you will have a suspension with 10 times fewer (one tenth as many) bacteria than in the 10⁻¹ tube or 100 times fewer organisms than in the starting, undiluted suspension (e.g., 1/10 x 1/10 = 1/100 dilution).

Continue making similar dilutions until you have the desired concentration (see Fig. 12).

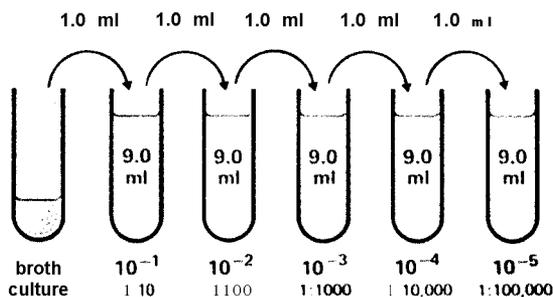


Figure 12. Diagram for tenfold dilutions.

7. **Inoculating biplates or quadrant plates of agar medium with a capillary pipette.**

When Middlebrook 7H- 10 or 7H- 11 agar is used for primary isolation of mycobacteria, the medium is usually placed in two-section plastic Petri dishes (**biplates**). Drug susceptibility tests of tubercle bacilli are usually done in 7H- 10 agar contained in four-sectioned plastic dishes (**quadrant plates**).

The capillary pipette often is used to place several drops of appropriate inoculum onto the agar medium in these sectioned dishes.

Again, using procedures described in 3. and 4., pp. 21-22, place an appropriate safety pipettor onto a sterile capillary pipette and select the tube of inoculum to be used for the sectioned dish.

Carefully remove the **screwcap** from the culture tube and flame the tube.

Expel some air from the capillary pipette by squeezing the bulb or rubber-tubing pipettor (a little experience will teach you how much the tubing or bulb must be squeezed for the small-volume capillary pipette). Place the pipette tip into the bacterial suspension and suck up the fluid. After removing the pipette from the test tube, flame and recap the tube, and replace it in the culture rack.

Using the thumb and middle finger of the left hand, remove the cover from the sectioned dish. **CAUTION:** Tilt the cover back toward yourself; if the cover is not held too far from the dish, it will act as a barrier to prevent airborne contaminants from impinging on the medium in the dish (see Fig. 13a).

Place the pipette tip at about a 45° angle and directly over the portion of medium to be inoculated (Fig. 13b).

Squeeze the bulb to release one free-falling drop of inoculum. To minimize aerosol, touch the drop of inoculum to the surface of the medium just before it drops free of the pipette tip (Fig. 13c).

Space out three such drops on the surface of the dish, as shown in Figure 13d. Each drop contains about 0.05 ml, so each section receives about 0.15 ml of inoculum. The drops may be left discrete and need not be spread to cover the surface.

Replace the cover of the dish and discard the used pipette into a pan containing disinfectant.

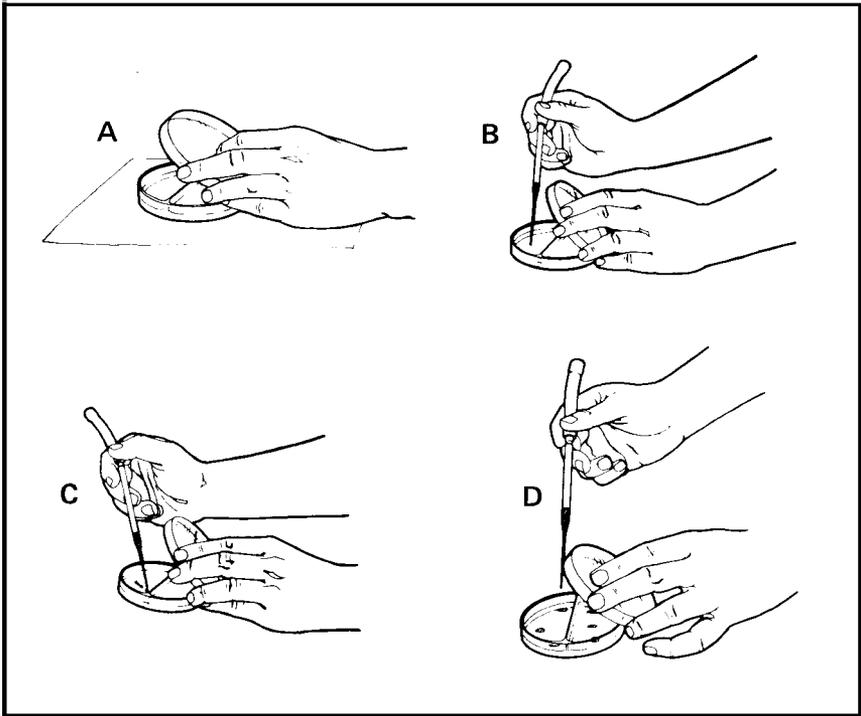


Figure 13. Inoculating plates of media with a capillary pipette

- A. Tilt the cover of the plastic dish back toward yourself:
- B. Pipette-ful of inoculum is held at 45° angle directly over portion of dish to be inoculated.
- C. Touch the drop of inoculum to the surface of the medium just before it drops free from the pipette.
- D. Three such drops of inoculum are usually placed on each section of the dish.

8. Spreading the inoculum over the surface of the medium

As an alternative to the procedure described in 7. (p. 29) personnel in some laboratories prefer full plates instead of sectioned dishes of medium, or they prefer to spread the inoculum over the entire surface of the medium.

In such cases, place the desired volume of inoculum on the surface to be inoculated.

A convenient “spreader” may be made (with a bit of practice) from a sterile capillary pipette (follow drawings in Fig. 14).

Remove a sterile capillary pipette from the pipette cannister and carefully insert **only the very tip** of the pipette into the blue cone of flame in the Bunsen burner so that the end of the pipette is sealed with a ball of melted glass (Fig. 14a).

Next make a bent “spreading arm” in the sealed pipette. The length of the “arm” depends upon the size of the dish being “spread.” If a full 100-mm dish is to be spread, make the arm about 40 mm long. If a biplate or quadrant dish is to be spread, make the arm only 20-25 mm long. These spreaders can be made easily and quickly, so an occasional “bad” spreader should be discarded and a new one made. Again, practice in preparing these spreaders is the key to a good product.

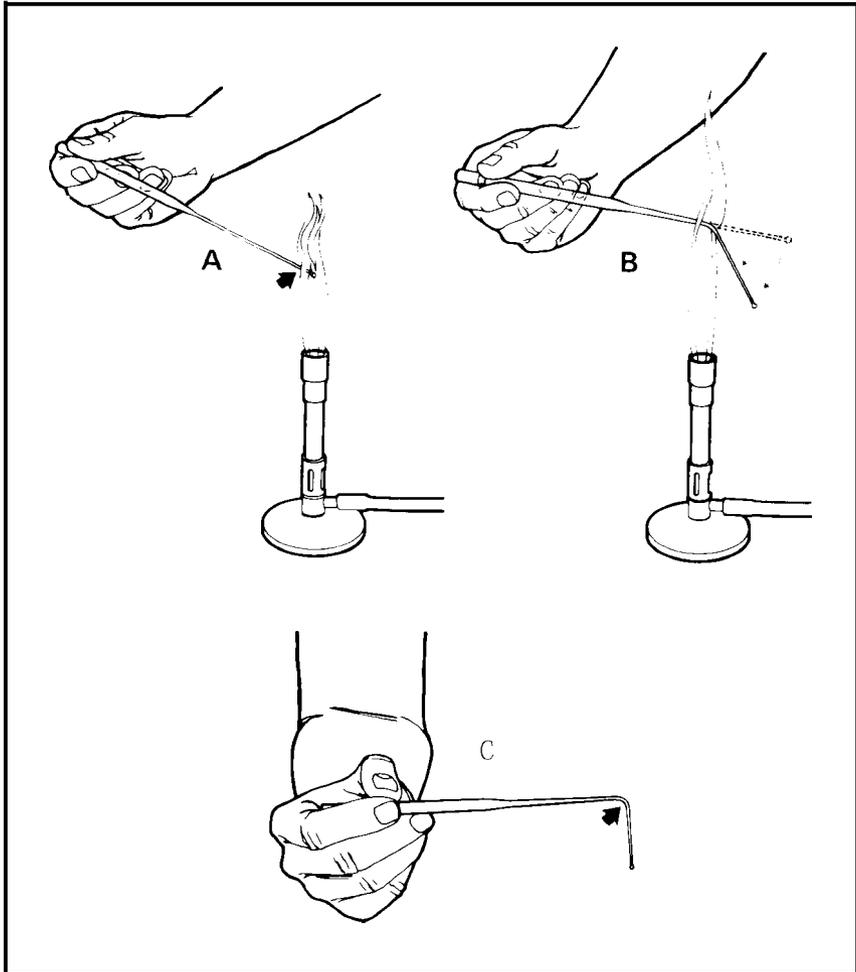


Figure 14. Preparation of a glass inoculum spreader.

- A. Heat seal the open end of a capillary pipette in the blue cone of a Bunsen burner flame.
- B. Hold pipette parallel to table top and heat at the point where bend is desired.
- C. When tubing starts to bend, remove from flame but continue to hold parallel to table top; if properly done, a bend of 90° is usually obtained.

Holding the pipette parallel to the counter top, place the capillary tubing in the tip of the blue cone of flame at the point where you want the tubing to bend (Fig. 14b).

The moment the tubing begins to bend, remove it from the fire, but continue to hold the pipette parallel to the counter top.

The weight of the hot, bending glass is usually enough to create an angle of almost 90° (see Fig. 14c).

Allow the hot tubing a few moments to cool, and then spread your inoculum over the surface of the medium.

Discard the bent spreader immediately after you have finished with it.

9. Inoculating the “spinner” plate

The “spinner” plate method is a fast way to spread an inoculum on the surface of a full (not sectioned) dish of medium, with the intention of obtaining isolated colonies for observation and/or selective picking. You will need a **Spray-Fisher** Petri dish turntable.

Place the dish of medium to be inoculated onto the dish turntable. Using aseptic procedure, as described in 3. and 4. (pp. 21-22), remove a loopful of inoculum from the bacterial suspension from which you would like to obtain a growth of isolated colonies. Hold the loop in the right hand.

Using the thumb and middle finger of the left hand, remove the lid from the dish, and with the ring finger of the same hand, give the turntable a sharp pull toward you to set it spinning counterclockwise (Fig. 15a).

The next procedure depends upon whether you wish to see isolated colonies in the center or at the periphery of the dish.

For isolated colonies in the center, touch the full face of the inoculating loop to the surface of the rotating plate at the outer edge (near 8 or 10 o'clock), and gradually move it toward the center of the dish (Fig. 15b). **NOTE:** the dish is rotating through all this procedure. The resulting inoculum is in the form of a gradually decreasing spiral.

When the loop reaches the center, lift it from the medium, stop the turntable, and replace the cover on the dish.

For isolated colonies at the periphery of the plate, touch the loop of inoculum to the middle of the rotating dish and push

it toward either 8 or 10 o'clock, lifting the loop just before it touches the edge of the dish (Fig. 15c).

Some culture suspensions (e.g., rapid growers) are extremely heavy and might still show confluent growth when inoculated as described above.

For heavy suspensions, dip the inoculating loop into the suspension, touch the loop to the side of the test tube to break the film of culture suspension, and then inoculate the spinning plate as described above, using this "empty" loop as inoculum.

For even heavier suspensions, touch the "empty" loop to the rotating plate with the edge of the loop rather than the full face.

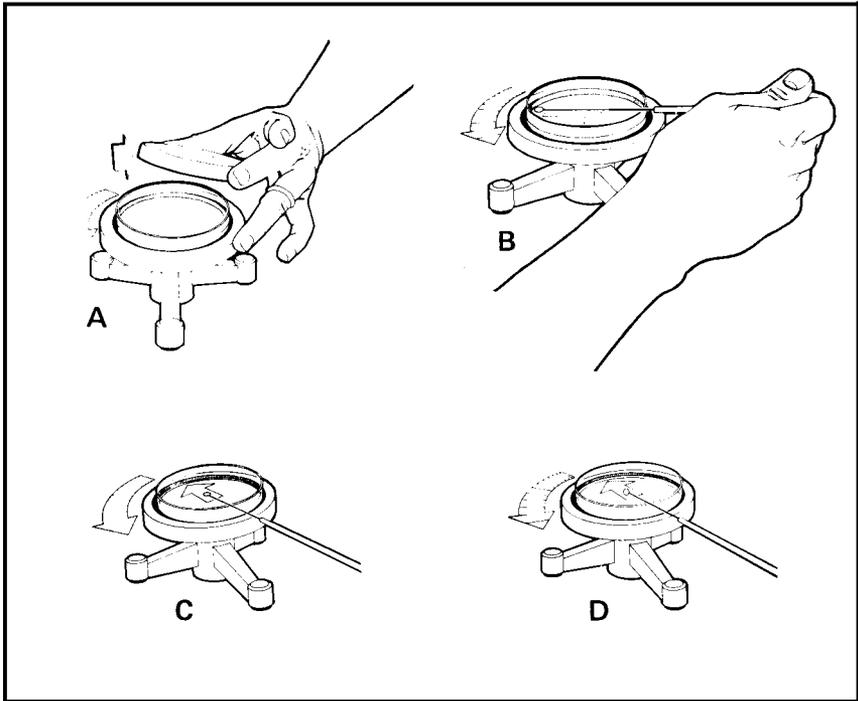


Figure 15. Inoculating the "spinner" plate.

- A. Remove lid from dish with thumb and middle finger of left hand; use "ring" finger to spin dish counterclockwise.
- B. To obtain isolated colonies in center, touch loop of inoculum to surface of rotating plate at outer edge (near 8- or 10- o'clock) and gradually move toward center. Lift loop when it reaches center of dish.
- C. To obtain isolated colonies near periphery, touch loop to center of rotating dish and push it toward 8- or 10- o'clock. Lift loop before it touches edge of dish.
- D. For heavy suspensions touch loopful of inoculum to inside wall of test tube to break film of culture suspension and/or inoculate plate with loop held on edge.

See Figure 15 for drawings of the methods described.

A properly "streaked" plate should have five to eight spiral turns on it. It takes some practice to have the dish rotating at the right speed and to move the loop across the medium at the right speed (not too rapidly) to get a good spiral. Once mastered, however, the technique is a great time saver.

REFERENCES

1. Center for Disease Control. 1976. Biological Safety Cabinet. Available by writing CDC. Atlanta, Ga. 30333
2. Hall, C.V. 1975. A biological safety centrifuge. *Hlth. Lab. Sci.* 12:104-106
3. Harrington, J.M. and H.S. Shannon. 1976. Incidence of tuberculosis, hepatitis, brucellosis, and shigellosis in British medical laboratory workers. *Brit. Med. J.* 1:759-762.
4. Kenny, M.T. and F.L. Sabel. 1968. Particle size distribution of *Serratia marcescens* aerosols created during common laboratory procedures and simulated laboratory accidents. *Appl. Microbiol.* 16:1 146-1 150.
5. Madsen, P.O. and R.E. Madsen. 1967. A study of disposable surgical masks. *Amer. J. Surg.* 114:43 1-435.
6. National Sanitation Foundation. 1976. Standard No. 49 for Class II (Laminar Flow) Biohazard Cabinetry. The National Sanitation Foundation. NSF Building. Ann Arbor. MI. 48105.
7. Pike, R.M. 1976. Laboratory-associated infections: summary and analysis of 3921 cases. *Hlth. Lab. Sci.* 13:105-114.
8. Reid, D.P. 1957. Incidence of tuberculosis among workers in medical laboratories. *Brit. Med. J.* 2:10-14.
9. Stern, E.L., J.W. Johnson, D. Veslcy, M.M. Halbrt, L.E. Williams, and P. Blumc. 1974. Aerosol production associated with clinical laboratory procedures. *Amer. J. Clin. Path.* 62:591-600.
10. National Institutes of Health. 1975. Certification of Class II (laminar flow) biological safety cabinets. U.S. Dept. Health and Human Services, PHS, NIH, National Cancer Institute, Office of Biohazards and Environmental Control. Bethesda, Maryland 20014.
11. National Institutes of Health. Formaldehyde decontamination of laminar flow biological safety cabinets. U.S. Dept. Health and Human Services, PHS, NIH, National Cancer Institute, Office of Biohazards and Environmental Control. Bethesda, Maryland 20014.
12. Center for Disease Control Publication. 1980. Proposed biosafety guidelines for microbiological and biomedical laboratories. CDC. Office of Biosafety, Atlanta. Georgia 30333.



II. COLLECTION AND
TRANSPORTATION OF SPECIMENS

II. COLLECTION AND TRANSPORTATION OF SPECIMENS

A. COLLECTION

The definitive diagnosis of tuberculosis (or other mycobacterioses) demands that tubercle bacilli (or other mycobacteria) be isolated from clinical specimens submitted to the laboratory for culture. The efficiency of any laboratory procedure used to culture mycobacteria from clinical specimens depends on the manner in which the specimen is obtained and handled afterwards. Therefore, specimens must be collected with the utmost care and promptly transported to the laboratory.

For optimal results, clinical specimens should be collected before chemotherapy is started, because even a few days of drug therapy may kill or inhibit sufficient numbers of bacilli to leave the bacteriologic confirmation of disease in doubt. Specimens must be collected in clean, sterile containers. To minimize the possibilities of saprophytic or nonviable mycobacteria being carried from one specimen to another, use one-use disposable plastic containers; if this is not possible, use glass containers which have been cleaned with dichromate sulfuric acid or other good cleaning solution.

Mycobacteria may localize in almost any organ of the body, so the laboratory may expect a variety of clinical materials to be submitted for examination. Because tuberculosis is primarily a pulmonary disease, most of these specimens will be secretions from the lungs.

The microbiologist should be prepared to process all intact specimens received, but he, or she, also should call the attention of attending medical personnel to inadequate specimens or possible reasons for negative culture reports (e.g., insufficient specimen, leakage in transit, and excessive delay in transport). The successful isolation of the causative agent of disease depends upon an adequate volume of specimen, properly collected and transported, and carefully processed.

The major kinds of specimens received in the laboratory will be discussed separately.

1. Sputum

A series of three to six single, early morning specimens (2,10) usually collected on successive days, are easy to handle in the laboratory and are less likely to be contaminated, especially if they are delivered by mail (7). In contrast, if the specimens are not mailed (i.e., if they are processed at the same institution in which they were collected), pooled sputum specimens

have been shown (9) to yield significantly more positive cultures than single morning collections. The same study showed however, that the single specimens yielded growth much earlier than the pooled specimens, so each kind of specimen has its advantages under appropriate circumstances. The choice of how many specimens to submit for initial diagnosis may be regulated by careful microscopic examination of smears prepared from digested, concentrated sputa. If smears are strongly positive, three specimens may be sufficient to confirm the diagnosis. The patient whose bacilli are difficult to detect on a smear should submit five or six, or even more, sputum specimens.

If mail-in specimens cannot be immediately shipped, they should be refrigerated to minimize overgrowth by undesirable contaminants. Specimens to be mailed must be carefully sealed and properly packaged to avoid leakage or breakage in transit (see II.B., p. 39).

Patients should be instructed in securing the most useful specimen, the naturally produced sputum. They should be told that nasopharyngeal discharge and saliva are **not** sputum. What is desired is the normally thick, yellowish (sometimes blood-tinged) exudative material brought up from the lungs after a deep, productive cough. The mouth should be rinsed free of food, residual mouthwash, and oral medications before sputum is collected. Specimens should only be collected into laboratory-approved containers clearly labelled with the patient's name and/or hospital number.

For both efficiency and proficiency in recovering mycobacteria, sputa should be collected and processed in the same container, and a 50-ml plastic, disposable centrifuge tube is recommended. Sputum volume should NEVER exceed one-fifth (10 ml) the volume of the collection container. This will permit adequate mixture of sputum with the digesting agent. It will also permit the digested sputum to be diluted enough to (i) minimize continued action of the toxic decontaminating agent and (ii) reduce the specific gravity of the digested sputum so that it can be more efficiently concentrated by centrifugation.

When the patient cannot expectorate or finds it difficult to do so, other methods may be used to collect pulmonary secretions:

- a. Sputum induction may be effected with warm, aerosolized hypertonic salt solution (1,4,5,1 1). Patients

generally prefer this to aspiration of gastric contents, and the microbiologist knows that sputum obtained by this method is "cleaner" than a specimen aspirated from the stomach. Induced specimens **should be so identified** on the laboratory request slip, because they resemble saliva.

- b. Laryngeal swabs (12,14) may be useful in patients who raise no sputum or swallow it, e.g., small children.
- c. Bronchoscopy and bronchial brushings may also be useful, not only for specimens collected at examination, but because the procedure itself often causes the patient to produce sputum naturally for several days. These latter specimens should also be processed.

2. Gastric lavage (4,5)

When sputum is difficult to obtain as described above, gastric lavage can perhaps be performed. It might be indicated for (i) patients with radiologic evidence of tuberculosis whose sputum is negative by other methods of examination; (ii) uncooperative patients who "raise no sputum" or who swallow it; (iii) patients who cannot expectorate because of other disorders, e.g., coma, neurological disease, etc.; (iv) patients suspected of submitting sputum other than their own; and (v) young children from whom sputum normally is difficult to obtain. The specimen is collected early in the morning before the patient has eaten, and preferably, while the patient is still in bed. Optimal results are attained when these specimens are collected from hospitalized patients, rather than when specimen is collected elsewhere and mailed to the laboratory. Likewise, patients should not be expected to travel long distances to hospital or clinic for collection of gastric lavage.

- a. Twenty to 50 ml of chilled, sterile distilled water passed into the stomach through a disposable plastic stomach tube should provide an adequate sample.
- b. The sample is recovered by using a sterile 50 ml syringe. It is placed in a sterile bottle or plastic tube.

The specimen should be processed promptly because mycobacteria die rapidly in gastric washings. If processing is to be delayed, the gastric lavage should be neutralized (13). For each 35 to 50 ml of gastric washings, use 1.5 ml of 40% anhydrous disodium phosphate (Na_2HPO_4) or two pH7.4 pHydrion buffer capsules or tablets.

Although both patients and microbiologists seem to prefer induced sputum to gastric lavage, a combination of aerosol induction of sputum followed in 30 minutes by gastric lavage has been shown (3) to yield more positive cultures than either method alone.

3. Urine

Before a urine specimen is collected, the external genitalia should be carefully washed. The specimen should be collected either as a single, early morning, voided midstream specimen (6), or as the total first morning specimen (8). Like the gastric lavage, urines are best collected and immediately processed in the hospital. Multiple specimens may have to be processed to demonstrate the presence of mycobacteria in the genitourinary tract, but this is far superior to the more cumbersome methods involved in handling the large volumes of 24-hour pooled samples.

4. Other body fluids

The physician aseptically collects such specimens as pleural, pericardial, spinal, synovial, and ascitic fluids, blood, bone marrow, and pus by using aspiration techniques or surgical procedure. Acid-fast bacilli are generally difficult to find in such specimens because they are so diluted by the large reservoir volume of the "infected" fluid. To improve chances of isolating mycobacteria from these specimens, laboratory personnel and the clinician must cooperate closely. For example, by prior arrangement with laboratory personnel, aseptically collected fluids may be placed immediately (i.e., at the bedside) into liquid medium (e.g., Middlebrook 7H-9, Dubos Tween albumin broth, Proskauer-Beck) in a ratio of 1 part fluid specimen to 5 (or 10) parts of liquid medium. Incubating such cultures at 35° to 37°C in 10% CO₂, with daily shaking, usually encourages the small numbers of mycobacteria to multiply. When weekly microscopic examination of stained smears from such cultures indicates the presence of acid-fast organisms, the liquid medium may be subcultured to solid media (egg or agar base) for growth and identification of the mycobacterial pathogen. If the aseptically collected fluid cannot be immediately inoculated into liquid medium, then sterile ammonium oxalate or heparin should be added to those fluids which may clot, and the specimens should be transported to the laboratory as quickly as possible.

If the collected specimens are not known to be sterile or if they are suspected of being contaminated they should be treated as described for sputum. If specimen volume permits, however, one portion should be inoculated to media without prior treatment and the other portion digested or decontaminated before it is inoculated onto media.

5. Tissues

Like body fluids, small pieces of tissue suspected of containing mycobacteria should be collected aseptically and placed in sterile containers without preservatives or fixatives. For shipment to distant laboratories by mail, the specimen should be protected from drying by adding sterile saline to cover the tissue; specimens should be maintained at 5° to 10°C or shipped in dry ice. If strict asepsis is observed in collecting and handling such specimens, they may be homogenized (only in a BSC) in sterile tissue grinders with a small amount of sterile saline or 0.2% bovine albumin and inoculated directly to liquid and solid media as described for body fluids. If the tissue is not known to be sterile, it may be homogenized first, with half of the homogenate being inoculated directly to media, and the other half treated as for sputum before being planted on media.

B. TRANSPORTATION

1. The shipment of diagnostic specimens such as urine, sputum, and tissue, and of cultures of etiologic agents, which includes all *Mycobacterium* spp., is subject to the packaging and labeling requirements of the Interstate Quarantine regulations (Federal Register, Title 42, Chapter 1, Part 72, revised July 30, 1972). Compliance with the requirements of the regulation is the responsibility of the shipper. Failure to comply with the applicable requirements subjects violators to penalty provisions which include fines and/or imprisonment.

Diagnostic specimens must be packaged to withstand leakage of contents, shocks, pressure changes, and other conditions incident to ordinary handling in transportation. The "ETIOLOGIC AGENT/BIOMEDICAL MATERIAL" label (described below) must not be affixed to shipments of diagnostic specimens.

2. Etiologic agents are subject to additional specific containment packaging and labeling requirements. Cultures of *Mycobacterium* should be shipped to reference laboratories

on solid medium in screw-cap tubes. Petri dish cultures and cultures in liquid medium must not be shipped.

Requirements for packaging etiologic agents are found in Section 72.25, paragraph (c) subparagraph (1) of the Interstate Quarantine regulations, as follows: Volume less than 50 ml. Material shall be placed in a securely closed, watertight container (primary container [test tube, vial, etc.]) which shall be enclosed in a second, durable watertight container (secondary container). Several primary containers may be enclosed in a single secondary container, if the total volume of all the primary containers so enclosed does not exceed 50 ml. The space at the top, bottom, and sides between the primary and secondary containers shall contain sufficient non-particulate material to absorb the entire contents of the primary container(s) in case of breakage or leakage. Each set of primary and secondary containers shall then be enclosed in an outer shipping container constructed of corrugated fiberboard, cardboard, wood, or other material of equivalent strength. See Figure 16.

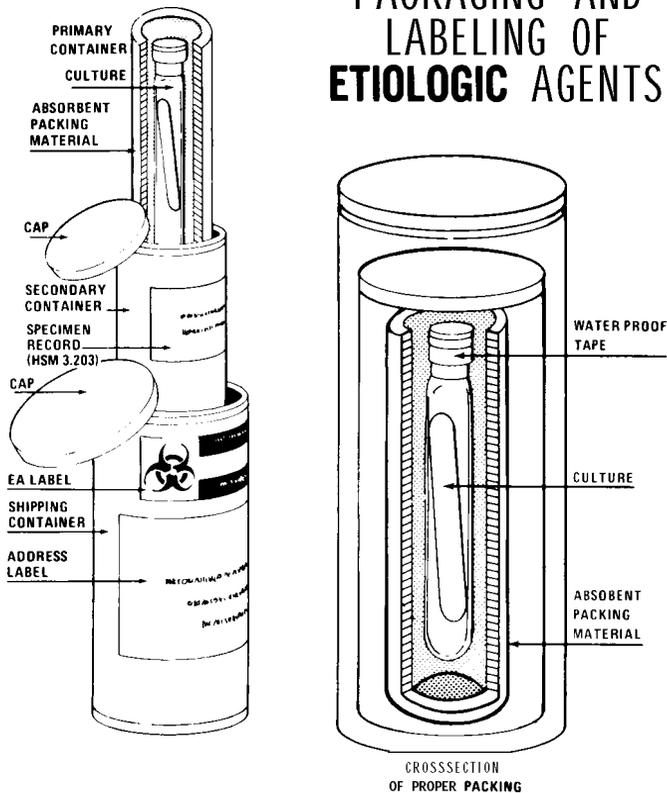
Subparagraph (2) describes packaging of volumes of 50 ml or greater. This description is similar to (1) except that the volume in a single primary container may not be over 500 ml and the total volume enclosed within the outer shipping container may not exceed 4000 ml.

Subparagraph (3) states that if dry ice is used as a refrigerant, it must be placed between the secondary and outer container in such a way that, as the dry ice sublimates, the secondary container does not become loose inside the outer container.

Department of Transportation regulations prohibit the shipment of more than 50 ml of an etiologic agent in a primary container on a passenger aircraft. A volume greater than 50 ml may be carried by air freight provided it is properly packaged and the total volume within a single outer container does not exceed 4000 ml.

An "ETIOLOGIC AGENT/BIOMEDICAL MATERIAL" label must be affixed on the outer container. These labels are available commercially (See Figure 16); they should measure 2 inches high by 4 inches long, and be printed in red on a white background. Shippers of etiologic agents must be familiar with the above regulations that govern packaging, labeling, and shipment of these products in interstate commerce.

PACKAGING AND LABELING OF ETIOLOGIC AGENTS



The Interstate Quarantine Regulations (Code of Federal Regulations, Title 42, Part 72.25, Etiologic Agents) was revised July 30, 1972, to provide for packaging and labeling requirements for etiologic agents and certain other materials shipped in interstate traffic.

The figure diagrams packaging and labeling of etiologic agents in volumes less than 50 ml in accordance with the provisions of subparagraph (C)(1) of the cited regulation.

The Etiologic Agents-Biomedical Material label (see subparagraph [C] [4] of regulations) which must be placed on all shipments of etiologic agents is depicted below. The label must be 2 inches high by 4 inches long and printed in red ink on white stock. For further information on any provision of this regulation contact:

Centers for Disease Control
Attn: Office of Biosafety
1600 Clifton Road
Atlanta, Georgia 30333
Telephone: (404) 329-3883

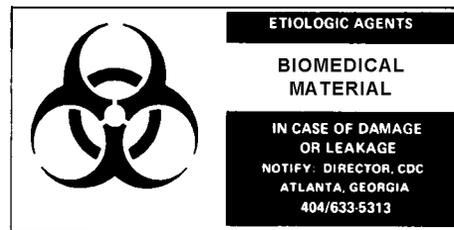


Figure 16.

Questions regarding this regulation should be addressed to:

Office of Biosafety
Centers for Disease Control
Atlanta, Georgia 30333
(Telephone 404/329-3883)

REFERENCES

1. Beck, G.J. and K. Nanda. 1962. Use of superheated aerosols as a diagnostic measure in tuberculosis. *Dis. Chest.* **42:74-78.**
2. Blair, E.B., G.L. Brown, and A.H. Tull. 1976. Computer files and analyses of laboratory data from tuberculosis patients. II. Analyses of six years' data on sputum specimens. *Amer. Rev. Respir. Dis.* **113:427-432.**
3. Carr, D.T., A.G. Karlson, and G.G. Stilwell. 1967. A comparison of cultures of induced sputum and gastric washings in the diagnosis of tuberculosis. *Mayo Clin.Proc.* **42:23-25.**
4. Elliott, R.C. and J. Reichel. 1963. The efficacy of sputum specimens obtained by nebulization versus gastric aspirates in the bacteriologic diagnosis of pulmonary tuberculosis. A comparative study. *Amer. Rev. Respir. Dis.* **88:223-227.**
5. Jones, F.L. Jr. 1966. The relative efficacy of spontaneous sputa, aerosol-induced sputa, and gastric aspirates in the bacteriologic diagnosis of pulmonary tuberculosis. *Dis. Chest.* **50:403-408.**
6. Kenney, M., A.B. Loechel, and F.J. Lovelock. 1960. Urine cultures in tuberculosis. *Amer. Rev. Respir. Dis.* **82:564-567.**
7. Kestle, D.G. and G.P. Kubica. 1967. Sputum collection for cultivation of mycobacteria: an early morning specimen or the 24-72 hour pool? *Amer. J. Clin. Path.* **48:347-349.**
8. Krasnow, I. 1978. Primary isolation of mycobacteria. *Lab. Med.* **9:26-31.**
9. Krasnow, I. and L.G. Wayne. 1969. Comparison of methods for tuberculosis bacteriology. *Appl. Microbiol.* **18:915-917.**
10. Kubica, G.P., W.M. Gross, J.E. Hawkins, H.M. Sommers, A.L. Vestal, and L.G. Wayne. 1975. Laboratory services for mycobacterial diseases. *Amer. Rev. Respir. Dis.* **112:773-787.**
11. Lillehei, J.P. 1961. Sputum induction with heated aerosol inhalations for the diagnosis of tuberculosis. *Amer. Rev. Respir. Dis.* **84:276-278.**
12. Tonge, J.I. and P.G. Hughes. 1956. A comparative study of laryngeal swabs and gastric lavage in the detection of tubercle bacilli. *Amer. Rev. Tuberc.* **73:930-939.**
13. Vandiviere, H.M., C.E. Smith and E.J. Sunkes. 1952. Evaluation of four methods of collecting and mailing gastric washings for tubercle bacilli. *Amer. Rev. Tuberc.* **65:617-626.**
14. Velu, S., S.S.L. Narayana, and T.V. Subbaiah. 1962. A comparison of the results of bacteriologic examination of a sputum collection and a pair of laryngeal swab specimens in patients receiving chemotherapy for pulmonary tuberculosis. *Tubercle* **43: 1-10.**

III. DEMONSTRATION AND ISOLATION OF MYCOBACTERIA



III. DEMONSTRATION AND ISOLATION OF MYCOBACTERIA

A. INTRODUCTION

The presence of mycobacteria in clinical specimens may be demonstrated either by examining stained smears through a microscope or by culturing the organisms on a variety of isolation media. The simplest procedure is microscopy. By far the fastest microscopic method is that of the direct smear in which selected cheesy, necrotic particles in the specimen are smeared onto the microscope slide, heat fixed, and then stained by using one of the procedures described later in this Section.

Because acid-fast bacilli cannot be specifically identified by smear examination and because many different species of mycobacteria are known to be potentially pathogenic for man, personnel in laboratories at Levels II and III must isolate the organism on some culture medium before they can identify it precisely. Except under unusual circumstances, personnel in most Level II laboratories will not make direct smears. Instead, they will make microscopic observations of concentrated sediments of clinical specimens that have been digested and decontaminated for culture inoculation. Therefore, methods for digesting and decontaminating specimens will be described first. The concentrated sediment obtained by using these procedures may be examined microscopically (III, D., p. 61) or inoculated onto isolation media.

Examination of the sources from which most clinical specimens suspected of containing mycobacteria are derived reveals an abundance of many contaminating microorganisms, most of them surrounded by organic debris (body fluids, white blood cells, red blood cells, tissue, etc.) which both maintains their viability and protects them from the harmful effects of decontaminating agents. Since these contaminating microbes generally reproduce much faster than mycobacteria, they will overgrow acid-fast organisms on culture media unless some attempt is made to inhibit or kill them.

The organic debris surrounding the organisms in a clinical specimen must therefore be liquefied, so that the decontaminating agents can kill the undesirable bacteria or fungi and the surviving mycobacteria can gain access to the nutrients of the medium onto which they are inoculated. Because mycobacteria are usually more resistant to the toxic effects of most chemical disinfectants than other microorganisms, chemical decontamination of diagnostic specimens is often successful.

An ideal digesting-decontaminating solution should:

- (1) Liquefy the organic debris in the specimen.
- (2) Kill contaminating microorganisms.
- (3) Not harm mycobacteria.

No one agent satisfies all three criteria, and investigators have been forced to make concessions in one or more areas in order to obtain mycobacterial growth on culture media.

Many digesting-decontaminating methods have been described, nearly all of which will kill a certain proportion of the mycobacteria in the specimen. To minimize the death of mycobacteria, the **step-by-step** procedure in all digestion/decontamination methods must be followed precisely. The mildest digestants which still effectively suppress contaminants should be used. Several of the more widely recommended digestion procedures are detailed here.

B. ISOLATION PROCEDURES

1. N-Acetyl-L-Cysteine-Sodium Hydroxide (NALC-NaOH) Method (8, 10).

This method is used at CDC. A mucolytic agent, N-acetyl-L-cysteine, is used for digestion and sodium hydroxide for decontamination. Sodium citrate is included in the digestant mixture because of its ability to bind, by chelation, heavy metal ions that may be present in the specimen and could inactivate the acetylcysteine (5).

a. Materials

N-acetyl-L-cysteine-NaOH (NALC-NaOH) solution, prepared as described below.

Tubes, 50 ml centrifuge, preferably plastic disposable
Media:

- (1) Agar base, e.g., Middlebrook 7H-10 or 7H-11
- (2) Egg base, e.g., Lowenstein-Jensen (L-J) or American Trudeau Society (ATS)

Microscope slides, frosted end

Phosphate buffered saline (0.067M), pH 6.8

Pipettes: 1, 5, 10 ml and capillary

Water, sterile distilled: (1) 30 to 40 ml/specimen

(2) 4.5 ml dilution blanks

Bovine albumin, 0.2% solution in saline.*

*Bovine albumin may be obtained from the Armour Co. or Pentax, Inc., Kankakee, Illinois. To prepare 2% solution, dissolve 0.85 g NaCl in 100 ml distilled water, add 2 g of bovine albumin fraction V and dissolve by swirling or gently stirring on magnetic stirrer. Adjust pH to 6.8 with 4% NaOH and sterilize by Seitz or membrane filtration. For 0.2% solution, dilute this 2% stock solution 1:10 with sterile saline.

b. Preparations

- (1) Prepare the volume of NALC-NaOH needed as indicated below:

Table 2. Preparation of N-acetyl-L-cysteine-sodium hydroxide solution

Volume of Digestant Needed (ml)	Mix indicated amounts (ml) of		Gm of NALC to Add
	4% NaOH ¹	2.9% sodium citrate ²	
50	25	25	0.25
100	50	50	0.50
200	100	100	1.00
500	250	250	2.50
1000	500	500	5.00

¹Add 4.0 g NaOH to 100 ml distilled water.

²Add 2.9 g sodium citrate to 100 ml distilled water.

The NaOH and sodium citrate may be prepared and sterilized (by autoclaving) in advance of mixing. Prepare only the volume of final digestant actually needed, because mucolytic activity of NALC is lost on standing (use within 18 to 24 hours).

- (2) Phosphate buffer solutions, 0.067M. Buffer solutions are prepared by mixing two stock solutions in proportions to give the required pH.

(a) Stock Solutions

(i) 0.067M disodium phosphate: In a volumetric flask dissolve 9.47 g of anhydrous Na_2HPO_4 in distilled water to make 1 liter (1,000 ml).

(ii) 0.067M monopotassium phosphate: In a volumetric flask dissolve 9.07 g of KH_2PO_4 in distilled water to make 1 liter (1,000 ml).

(b) pH 6.8 Buffer Solution

Mix 50.0 ml of (i) with 50 ml of (ii).

Check pH on meter or with pH test paper.

(c) **pH 7.0 Buffer Solution**

Mix 6 1.1 ml of (i) with 38.9 ml of (ii).
Check **pH** on meter or with **pH** test paper.

(d) **pH 6.6 Buffer Solution**

Mix 37.5 ml of (i) with 62.5 ml of (ii).
Check **pH** on meter or with **pH** test paper.

- (3) Label media, slides and tubes with appropriate data, e.g., patient's name, or hospital number, or specimen accession number. Media to be inoculated should show date of inoculation.
- (4) Assemble supplies and place on cart or table near door to isolation room.
- (5) Don protective clothing, enter isolation room, and transfer supplies onto work area.

c. **Procedures**

Sputum (natural or induced): 90% to 95% of all specimens submitted to the laboratory for diagnosis of tuberculosis are sputa.

- (1) Combine the desired amounts of **NaOH-Na citrate** solution (see table on page 45), add the appropriate amount of **NALC** powder and mix.
- (2) Line up specimens in numerical order and transfer sputum to centrifuge tubes. Do not add more than 10 ml sputum to a 50 ml centrifuge tube. Add to the specimen a volume of **NALC-NaOH** solution equal to the volume of the specimen.
- (3) Tighten cap of tube and mix by swirling on a test tube mixer until liquefied (usually 5 to 20 seconds). Avoid extreme agitation or frothing because this will oxidize and inactivate the acetylcysteine; the centrifugal mixing afforded by the test tube mixer is ideal. The height which the swirling mixture of sputum and digestant reaches when placed on a test tube mixer can be controlled by proper placement of fingers on the tube to be mixed and by regulating tube pressure on the rotating head of the mixer. Figure 17 shows the proper way to hold the tube.

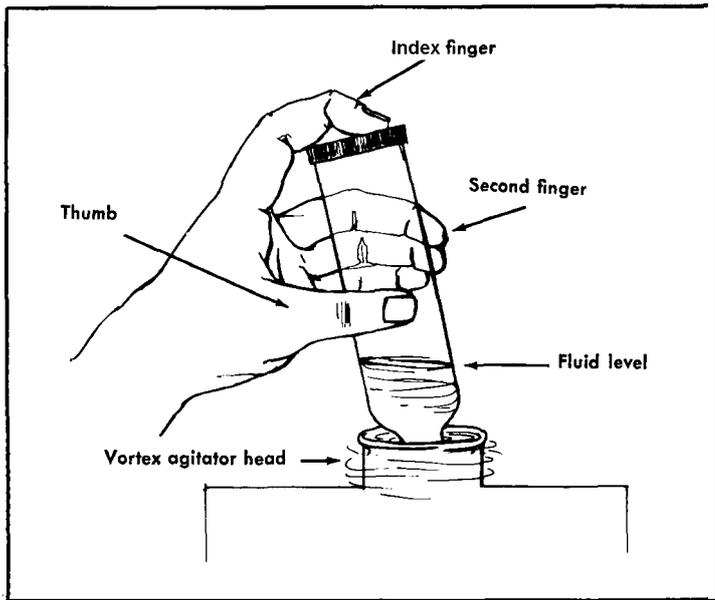


Figure 17. Proper use of test-tube mixer.

- (4) Let stand for 15 minutes (not over 20 minutes) at room temperature to decontaminate. If more active decontamination is desired, increase the concentration of NaOH in the above table to 5% or 6% instead of increasing the time the specimen is exposed to digestant.
- (5) Fill centrifuge tubes to 50-ml level with sterile distilled water or phosphate buffer, pH 6.8 (0.067M). Tighten screwcap and swirl by hand to mix.
- (6) Centrifuge at 2,000 to 3,000 x g for 15 minutes using aerosol-free, sealed centrifuge cups. *
The rpm required to produce 2,000 to 3,000 x g will vary with the centrifuge and the radius of the rotating head.
- (7) Decant the supernatant fluid into a discard can containing disinfectant, flame the lip of the tube (or swab with disposable gauze pledget soaked in disinfectant), and replace the cap.

*If aerosol free cups are not available, a centrifuge may be modified to minimize or eliminate aerosols from broken or leaking tubes by installing an absolute filter and air evacuating pump on the centrifuge bowl (3).

- (8) Add 1 to 2 ml of sterile 0.2% bovine albumin fraction V (see p. 44) with a sterile pipette. Shake gently to resuspend the sediment; this final suspension does not need to be neutralized. If sediment will be inoculated immediately onto media, sterile water or saline may be used in place of 0.2% bovine albumin.
- (9) Mix sediment with a sterile 1 ml pipette and make 1:10 dilution (Fig. 18) by adding 0.5 ml of resuspended sediment to 4.5 ml sterile water (or 1 ml to 9.0 ml). Mix with the pipette or on vortex mixer with caps tight. Diluting the resuspended sediment decreases the concentration of any toxic substances which may inhibit growth of mycobacteria.
- (10) Place 0.1 ml (two or three drops) of the 1: 10 dilution onto the surface of half of a biplate of 7H- 10 medium.
- (11) Place two drops of the 1: 10 dilution onto the surface of each of the two tubes of Lowenstein-Jensen medium and spread the inoculum over the surface of the medium.
- (12) Place two drops of the undiluted sediment onto the other half of the 7H- 10 biplate.

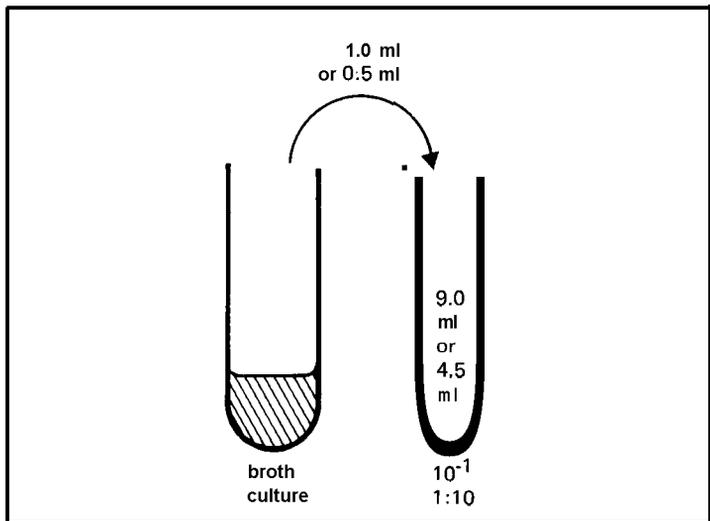


Figure 18. Scheme for making ten-fold serial dilution.

- (13) Place two drops of the undiluted sediment onto each of two tubes of Lowenstein-Jensen (LJ) medium and spread the inoculum over the surface of the medium.
- (14) With a bent glass rod (see I F.8., p. 30), spread the inoculum over the surface of the 7H-10 plates (the 1: 10 dilution first, then the undiluted inoculum).
- (15) Make two smears of the undiluted sediment by spreading a drop over an area 1 x 2 cm on the microscope slide (See D., p. 61 for details).
- (16) Place slides on the electric slide warmer at 65" to 70°C for 2 hours (or overnight) to heat fix.
- (17) Place each 7H-10 plate in an individual CO₂-permeable polyethylene bag and incubate under 10% CO, at 35° to 37°C.
- (18) Incubate the LJ medium at 35" to 37°C with tubes in a horizontal position and caps loosened. Incubation in an atmosphere of CO, will encourage earlier growth.
- (19) If direct drug tests are to be performed, refrigerate the remaining undiluted sediment until ready to use.

OTHER LESS COMMONLY ENCOUNTERED SPECIMENS THAT MAY BE CULTURED FOR MYCOBACTERIA.

GASTRIC LAVAGE*

Mucoid gastric lavage

- (1) Add 50 to 100 mg ("pinch") of NALC powder to 20 to 50 ml of gastric lavage.
- (2) Mix by hand or with test tube mixer as for sputum.
- (3) Centrifuge at 2,000 to 3,000 x g for 30 minutes.
- (4) Decant supernatant fluid and resuspend sediment in 2 to 5 ml of sterile distilled water.
- (5) Add an equal volume of NALC-NaOH and proceed as for sputum.

*These specimens should be processed within 4 hours of collection.

Fluid gastric lavage

- (1) Centrifuge entire specimen for 30 minutes.
- (2) Decant and resuspend in 2 to 5 ml of sterile distilled water. Pool sediments if more than one container is used for centrifugation.
- (3) Add an equal volume of NALC-NaOH and proceed as for sputum.

LARYNGEAL SWAB

- (1) With sterile forceps, transfer the swab to a sterile centrifuge tube.
- (2) Add 2 ml sterile distilled water or saline.
- (3) Add 2 ml NALC-NaOH.
- (4) Swirl on test tube mixer.
- (5) Let stand 15 minutes to decontaminate.
- (6) With sterile forceps, remove swab from centrifuge tube.
- (7) Fill the tube with sterile buffer or water and proceed as for sputum.

TISSUE

- (1) Grind with sterile tissue grinder in sterile 0.85% saline or 0.2% bovine albumin. When tissue (e.g., from the lung) contains mucus, add a pinch of N-acetyl-L-cysteine.
- (2) If tissue is collected and processed aseptically, inoculate directly to both liquid and solid media.
 - (a) Smear liquid culture once a week until acid-fast bacilli are seen microscopically. Inoculate solid media with this liquid culture as soon as smear is positive.
 - (b) If smear still is not positive after 6 to 8 weeks, streak each of four egg slants and two 7H-10 plates with 0.1 ml or more of the broth culture and incubate at 35° to 37°C for 4 to 8 weeks.
- (3) When tissue is not collected aseptically, place homogenate from (1) above into a tube and process as sputum.

BLOOD

- (1) Add 10 ml aseptically collected blood to 50 ml of a Tween liquid medium, such as Dubos or Middlebrook 7H-9.
- (2) Incubate at 35° to 37°C.
- (3) Smear this liquid culture once a week. Stain, and examine smears under the microscope for presence of acid-fast bacilli.
- (4) Inoculate solid media with any smear-positive liquid cultures.

OTHER BODY FLUIDS

Muco-purulent materials

- (1) Handle as for sputum when volume is 10 ml or less.
- (2) Handle as for mucoid gastric lavage when volume is more than 10 ml.

Fluid materials

- (1) If collected aseptically, centrifuge and inoculate sediment directly onto culture media.
- (2) If not aseptically collected:
 - (a) Handle as for sputum when volume is 10 ml or less.
 - (b) Handle as for fluid gastric lavage when volume is more than 10 ml.

2. Sodium Hydroxide (NaOH) Method (7, 8)

Sodium hydroxide is extremely toxic for mycobacteria as well as contaminants; therefore, care must be exercised in the use of this procedure, and prescribed time limits should be strictly adhered to.

a. Materials

Sodium hydroxide, pellets

2.0 N HCl

Hydrochloric acid-phenol red indicator

Tubes, 50 ml centrifuge, preferably plastic disposable.

b. Preparation

2% NaOH: 2 g per 100 ml distilled water

4% NaOH: 4 g per 100 ml distilled water

- (1) Select the desired concentration of NaOH (the lowest concentration compatible with effective digestion and decontamination should be used), prepare in volume, and sterilize by autoclaving.
- (2) Prepare the 2.0 N HCl by **carefully** adding 33 ml concentrated HCl to 200 ml distilled water and sterilize by autoclaving.
- (3) Prepare the HCl-phenol red indicator by combining 20 ml of phenol red solution* with 85 ml concentrated HCl and enough distilled water to make 1,000 ml. The phenol red indicator may also be used separately.

c. Procedure

- (1) Add an equal volume of the appropriate NaOH solution to the specimen contained in a 50 ml centrifuge tube and tighten cap.
- (2) Shake vigorously on a test-tube mixer until effectively digested. Let stand for 15 to 20 minutes. The paint conditioning machine is still used in some laboratories to shake NaOH-treated specimens. The main reason for discouraging its use is that the danger of tubes breaking and aerosol being disseminated is greater with it than with the the test-tube mixer.
- (3) Centrifuge the specimen for 15 minutes at 2000 to 3000x g.
- (4) Decant the supernatant fluid into a splash-proof container and neutralize the sediment with HCl by either:
 - (a) Adding the HCl -phenol red indicator drop by drop until the sediment turns a persistent yellow, or
 - (b) Adding a drop of the phenol red indicator and then adding HCl drop by drop until the sediment turns a persistent yellow.

* 80mg phenol red powder in 20ml 4% NaOH

- (5) Resuspend the sediment with 1 to 2 ml of sterile water (sterile solutions of saline or 0.2% bovine albumin may be used) and inoculate desired media.

3. Zephiran-Trisodium Phosphate (Z-TSP) Method (10, 11)

In this technique (Z-TSP), trisodium phosphate is used to homogenize the specimen, and zephiran (benzalkonium chloride) is used to control contaminants. Zephiran is bacteriostatic to mycobacteria unless it is neutralized. The phospholipid of egg medium effectively neutralizes most of this quaternary ammonium compound, but most clear agar media do not have such a "built-in" neutralizer. Therefore, to insure growth on agar medium (e.g., 7H-10 or 7H-11) of mycobacteria in Z-TSP-digested specimens, the digested, centrifuged sediment must be washed with buffer before the agar medium is inoculated (4).

a. Materials

Zephiran-trisodium phosphate (Z-TSP)
Neutralizing buffer, pH 6.6

b. Preparation

- (1) Z-TSP. Dissolve 1 Kg of trisodium phosphate ($\text{Na}_3\text{PO}_4 \cdot 12\text{H}_2\text{O}$) in 4 liters of hot distilled water. To this, add 7.5 ml concentrated Zephiran (17% benzalkonium chloride, Winthrop Laboratories), mix well, and store at room temperature.
- (2) Neutralizing buffer, pH 6.6 (see (d), p. 46).
 - (a) Mix 37.5 ml of stock buffer (i) and 62.5 ml of stock buffer (ii). Check on pH meter. Autoclave and store at room temperature, or
 - (b) Use neutralizing buffer (Difco).

c. Procedure

- (1) Add to the specimen a quantity of Z-TSP equal to the volume of the specimen.
- (2) Tighten screwcap securely and agitate vigorously on a shaking machine for 30 minutes.
- (3) Let the specimen stand 20 to 30 minutes without further agitation.

- (4) Transfer specimen to 50-ml screwcap centrifuge tube and centrifuge at 2,000 to 3,000 x g for 20 minutes.
- (5) Decant the supernatant fluid into a splash-proof container and resuspend the sediment in 20 ml neutralizing buffer, pH 6.6, and mix well.
- (6) Centrifuge the specimen again for 20 minutes, and discard the supernatant fluid into a splash-proof container, retaining some fluid to resuspend the sediment.
- (7) Use a sterile capillary pipette to mix the sediment and inoculate three drops onto:
 - (a) Each tube of egg medium.
 - (b) Each plate of agar medium.
- (8) Spread inoculum over surface of medium and incubate egg medium horizontally at 35°C and agar medium in 10% CO₂ at 35°C.

4. **Cetylpyridinium Chloride-Sodium Chloride (CPC-NaCl) Method (9).**

This method (CPC-NaCl) was proposed as a means of decontaminating sputa in transit, so that upon arrival in the laboratory, the specimens would need only to be concentrated before being inoculated onto media. Preliminary observations suggest that this method (i) provides a significant decrease in the laboratory time required for processing sputa, (ii) increases the number of positive cultures of mycobacteria (especially bacilli other than *M. tuberculosis*), and (iii) results in less contamination than the NALC-NaOH method.

Because cetylpyridinium chloride is bacteriostatic to mycobacteria on agar base medium and because questions as to the effectiveness of neutralizing buffer have not been resolved, only egg base medium is inoculated in this procedure. The NaCl liquefies and the CPC decontaminates the specimen.

a. **Materials**

Cetylpyridinium chloride, 1%

Sodium chloride, 2%

Centrifuge tubes, 50 ml, preferably plastic disposable.

Sterile water, 20 to 30 ml per specimen.

Sterile saline or 0.2% bovine albumin fraction V, 1 to 2 ml per specimen.

b. Preparation

- (1) **CPC-NaCl.** Dissolve 10 g cetylpyridinium chloride and 20 g of NaCl in 1 liter of distilled water. The solution is self-sterilizing and remains stable for extended periods at room temperature if caps are kept tight and the solution is protected from light and excessive heat. Crystals that may form in the working solution may be dissolved with gentle heat.
- (2) 0.2% bovine albumin fraction V (see footnote, p. 44).

c. Procedure

- (1) Collect sputa in 50-ml screwcap centrifuge tubes.
- (2) Add an equal volume of the **CPC-NaCl** solution, securely tighten caps, and shake by hand (inside biological safety cabinet) until specimens become fluid.
- (3) Package specimens in double mailing containers and send to the laboratory for processing. The specimen is digested and decontaminated in transit.
- (4) After receiving the specimen in a laboratory, fill each sputum container to the 50-ml mark with sterile distilled water, replace the cap, and tighten securely.
- (5) Centrifuge the specimen at 2,000 to 3,000 \times g for 20 minutes.
- (6) Decant supernatant fluid into splash-proof container and suspend the sediment in 1 to 2 ml sterile water, saline, or 0.2% bovine albumin fraction V.
- (7) Inoculate resuspended sediment onto egg medium.
- (8) Incubate medium at 35° to 37°C.

5. Oxalic Acid Method (2, 10).

This method is used to process specimens from patients whose cultures are consistently contaminated with *Pseudomonas* species.

a. **Materials**

- 5% Oxalic acid
- 4% Sodium hydroxide
- Physiological saline
- Indicator solution, phenol red

b. **Preparation**

- (1) Oxalic acid ($C_2H_2O_4 \cdot 2H_2O$): mix 5 g per 100 ml distilled water and autoclave.
- (2) Sodium hydroxide: Mix 4 g per 100 ml distilled water and autoclave.
- (3) Physiological saline: Mix 0.85 g per 100 ml distilled water and autoclave.
- (4) Phenol red indicator: Dissolve 80 mg phenol red powder in 20 ml 4% NaOH. When solution is complete, bring volume up to 1,000 ml.

c. **Procedure**

- (1) Add an equal volume of 5% oxalic acid to each specimen.
- (2) Mix on test-tube mixer. Let stand for 30 minutes with occasional shaking.
- (3) Add sterile saline to lower the specific gravity and dilute the acid.
- (4) Centrifuge the specimen at 2,000 to 3,000 x g for 20 minutes.
- (5) Decant the supernatant fluid into splash-proof container.
- (6) Add a few drops of the phenol red indicator to the sediment and neutralize with the 4% NaOH until the first persistent pale pink color forms.
- (7) Inoculate media and incubate at 35" to 37°C.
- (8) Make a smear and stain for acid-fast bacilli.

6. Sulfuric Acid Method (10,12).

When urine specimens processed by other methods consistently yield contaminated cultures, the sulfuric acid method may provide satisfactory culture results.

a. Materials

- 4% Sulfuric acid
- 4% Sodium hydroxide
- Sterile distilled water
- Indicator, phenol red

b. Preparations

- (1) Sulfuric acid (H_2SO_4): Add slowly, while stirring, 40 ml of concentrated sulfuric acid to 960 ml of distilled water. Store at 4°C .
- (2) Sodium hydroxide: Dissolve 4 g in 100 ml distilled water and autoclave.
- (3) Phenol red indicator: Dissolve 80 mg phenol red powder in 20 ml 4% NaOH. When solution is complete, bring volume to 1,000 ml.

c. Procedure

- (1) Centrifuge entire specimen for 30 minutes at 2,000 to 3,000 x g. This may require several tubes.
- (2) Decant supernatant fluid and consolidate sediments. If volume is small, add 1 or 2 ml of sterile water.
- (3) Add an equal volume of 4% H_2SO_4 to the sediment.
- (4) Mix and allow to stand for 15 minutes.
- (5) Fill the tube to the 50-ml mark with sterile water.
- (6) Centrifuge at 2,000 to 3,000 x g for 15 minutes. Decant supernatant fluid into splash-proof container.
- (7) Add to the sediment a few drops of the phenol red indicator solution and neutralize with 4% NaOH until the first pale pink color forms.
- (8) Inoculate and incubate media at $35''$ to 37°C .
- (9) Make a smear and stain for acid-fast bacilli. Although earlier literature may discourage this, the observation of large numbers of AFB in stained smears is usually evidence of infection. The detection of only rare AFB is of doubtful or no significance.

C. INCUBATION OF CULTURES

1. Plates of inoculated media may stay for a few hours at room temperature before they are placed in CO₂ permeable polyethylene plastic bags and sealed for incubation. Keep shielded from light (6).
2. Temperature: 35° ± 2°C, not to exceed 37°C. All cultures from skin lesions must also be incubated at a temperature not to exceed 33°C, because such cultures may contain *M. marinum* or *M. ulcerans* which grow better at the lower temperature.
3. Atmosphere: 5% to 10% carbon dioxide in air (1).
4. Medium in tubes:

Place in a slanted position with the screwcap loose for at least 1 week to permit exposure to carbon dioxide and consequent stimulation of growth. If necessary, then tighten caps to prevent dehydration, and stand tubes upright if space is a problem.
5. 7H-10 medium in plates:

Place plates medium-side down in a plastic bag; polyethylene allows carbon dioxide to pass into the bag. Seal the bag with tape or a heat-sealing apparatus, or fold the top over two or three times and staple. Squeeze the air out of the bag before sealing.
6. If excess moisture accumulates during incubation, some of the following suggestions may be helpful:
 - a. Avoid inoculating a wet medium surface. Take plates out of the refrigerator on the day before they are to be inoculated and leave them at room temperature in a clean, dark area.
 - b. After inoculating the media, shield the plates from contaminating air currents and direct or indirect daylight. Leave the plates for several hours before placing them in the incubator. This permits most of the inoculum fluid to evaporate or to be absorbed.
 - c. During incubation:
 - (1) Incubate plates in individual bags. This facilitates weekly reading of plates. Furthermore, moisture tends to accumulate in the plate on the bottom of the pile, and rotation helps prevent this.

- (2) If several plates are incubated in a large bag, stack them no more than six high. Pressure of the upper plates may “seal” moisture in lower plates and also may interfere with the circulation of CO₂.
 - (3) Incubate plastic plates on an “insulating” surface. Plastic plates in direct contact with metal shelves seem to collect more moisture than those “insulated” from the metal shelving. Styrofoam (1/2 inch) and corrugated cardboard are satisfactory insulators.
 - (4) Avoid incubating plates on shelves adjacent to the door. Alternating warm and cool air currents, created by opening and closing the door, cause moisture to collect within the plastic bags.
 - (5) Make sure that the air within the incubator is circulating properly.
- d. When reading plates stacked in large plastic bags, open the bags immediately after removing them from the incubator. Plates incubated singly in transparent plastic bags may be read without opening the bag.
 - e. Condensate on the inside of plate lids interferes with reading cultures. To avoid this, have a warming pad* adjacent to the microscope and place plates on it lidside down.
7. For maximum growth on culture media:
- a. Follow directions precisely during decontamination procedure. Overexposure to the chemicals may “damage” mycobacteria and retard their growth.
 - b. Make sure pH is balanced. An imbalance may be due to:
 - (1) Failure to centrifuge sufficiently to pack the sediment so that all the supernate can be decanted, or
 - (2) A too heavy inoculum, which interferes with the buffering action of the medium.

*An electric heating pad is satisfactory.

- c. Make sure the supply of CO₂ in the incubator is sufficient.
 - (1) The recommended concentration of CO₂ is 5% to 10%. Concentrations above 40% may be lethal to mycobacteria.
 - (2) Test the atmosphere of the incubator daily with a Fyrite CO₂ gas analyzer, or some other means of determining the concentration of CO₂.
 - (3) Be certain the plastic bags in which the plates are incubated are of polyethylene, which permits CO₂ to circulate; Mylar bags are **not** CO₂ permeable.
 - (4) Many strains of mycobacteria are dependent upon CO₂, and will not grow without it.

- d. In preparing media:
 - (1) Use good quality ingredients and follow directions.
 - (2) Do not overheat medium during preparation. Monitor temperature of autoclaves and inspissators.
 - (3) Do not expose the medium to direct daylight during or after preparation. This is especially important for 7H-10 and 7H-11 (6).
 - (4) Make sure plates of agar medium contain 20 ml; otherwise, the medium may be so thin that it dries out during storage or incubation.

- e. Since some strains of mycobacteria grow more slowly than others and may need to be incubated a week or two longer than most to produce visible colonies, do not discard cultures as negative until after at least 6 to 8 weeks.

REFERENCES

1. Beam, R.E., and G.P. Kubica. 1960. Stimulatory effects of carbon dioxide on the primary isolation of tubercle bacilli on agar containing medium. *Amer. J. Clin. Path.* **50**:395-397.
2. Corper, H.J., and N. Uyei. 1930. Oxalic acid as a reagent for isolating tubercle bacilli and a study of the growth of acid-fast nonpathogens on different mediums with their reaction to chemical reagents. *J. Lab. Clin. Med.* **15**:348-369.
3. Hall, C.V. 1975. A biological safety centrifuge. *Health Lab. Sci.* **12**: 104-106.

4. Krasnow, I., and G.C. Kidd. 1965. The effect of a buffer wash on sputum sediments digested with zephiran trisodium phosphate on the recovery of acid-fast bacilli. *Amer. J. Clin. Path.* **44**:238-240.
5. Kubica, G.P., A.J. Kaufman, and W.E. Dye. 1964. Comments on the use of the new mucolytic agent, N-acetyl-L-cysteine, as a sputum digestant for the isolation of mycobacteria. *Amer. Rev. Respir. Dis.* **89**:284-286.
6. Miliner, R.A., K.D. Stottmeier, and G.P. Kubica. 1969. Formaldehyde: a photothermal activated toxic substance produced by Middlebrook 7H-10 medium. *Amer. Rev. Respir. Dis.* **99**:603-607.
7. Petroff, S.A. 1915. A new and rapid method for the isolation and cultivation of tubercle bacilli directly from the sputum and feces. *J. Exp. Med.* **21**:38-42.
8. Runyon, E.H., A.G. Karlson, G.P. Kubica, and L.G. Wayne. 1974. In E.H. Lennette, E.H. Spaulding, and J.P. Truant, (ed.) *Manual of Clinical Microbiology*. 2nd ed. American Society for Microbiology, Washington, D.C., p.150.
9. Smithwick, R.W., C.B. Stratigos, and H.L. David. 1975. Use of cetylpyridinium chloride and sodium chloride for the decontamination of sputum specimens that are transported to the laboratory for the isolation of *Mycobacterium tuberculosis*. *J. Clin. Microbiol.* **1**:41 I-413.
10. Vestal, A.L. 1975. Procedures for the Isolation and Identification of Mycobacteria. DHEW No. (CDC) 75-8230, Center for Disease Control, Atlanta, Georgia 30333.
11. Wayne, L.G., I. Krasnow, and G. Kidd. 1962. Finding the "hidden positive" in tuberculosis eradication programs. *Amer. Rev. Respir. Dis.* **86**:537-54 I.
12. Willis, H.S., and M.M. Cummings. 1952. *Diagnostic and Experimental Methods in Tuberculosis*. 2nd. ed. Chas. C. Thomas, Springfield, Ill, p.99.

D. ACID-FAST MICROSCOPY

The observation of acid-fast bacilli in stained smears examined under the microscope may be the first bacteriologic evidence that mycobacteria are present in a clinical specimen.

Although smear examination is the least sensitive method for detecting mycobacteria and does not permit the observed organisms to be specifically identified, it is the easiest and most rapid procedure that can be performed in the laboratory, and it can be helpful in several ways: (1) it provides a presumptive diagnosis of mycobacterial disease; (2) it makes it possible to rapidly identify most infectious cases (i.e., those smear positive); (3) it can confirm that cultures growing on media are indeed acid-fast; (4) it may be used to follow the progress of tuberculosis patients on chemotherapy; and (5) it is useful in determining appropriate dilutions of sediments for direct drug susceptibility tests.

General comments and precautions.

Microscope slides used for acid-fast staining should be new, clean, and unscratched. Acid-fast material from previous smears may be retained (e.g., in scratches) on old, washed, and reused slides, and thus may lead to false positive reports.

One end of the slide should be labeled with the patient's name and/or hospital identification number, and date.

The specimen to be smeared should be spread over an area approximately 2 cm square; the 1 x 2-cm rectangle below illustrates the size.



Specimen material may be smeared over a larger area, but this would be self-defeating. If a microscopist examines 100 to 300 fields on a stained smear 1 x 2 cm, he or she only views 1 /100 to 4/1 00 of the entire area of the smear (10,19). A larger smear, examined in the same manner, would further reduce examining efficiency. Keep the area of the smear fairly small; 1 x 2 cm is satisfactory.

Use a bacteriological loop, applicator stick, swab, or pipette to make the smear. The loop may be reused after it is dipped into an alcohol sand flask and sterilized in the flame of a Bunsen burner, but applicator sticks, swabs, and pipettes should be discarded after each smear is prepared.

Let the smear air dry, and then heat fix it. The smear may be heat fixed on an electric slide warmer at 65° to 75°C for at least 2 hours (overnight is acceptable), or it may be passed 3 to 4 times through the blue cone of a burner flame as for other bacteriological smears.

Heat fixing does not always kill the mycobacteria, so be careful while handling smears. Always discard stained slides into covered pans containing disinfectant solution and autoclave before disposal, or discard into 3% amphyl solution overnight. Many acid-fast staining procedures have been described, including hot and cold staining methods, and those which employ fluorochrome dyes. Some work well and others are less satisfactory, but all are based on the property of mycobacteria to retain the primary stain, even after being exposed to strong mineral acids or acid-alcohol—thus the term “acid-fast bacilli.”

Care must be exercised in preparing and staining smears to avoid such problems as poorly stained preparations and false positive or false negative reports. The following suggestions may be helpful in avoiding some of these problems.

1. Avoid insufficient destaining with acid-alcohol.
 - a. Most mycobacteria are strongly acid fast and are not easily decolorized.
 - b. Smears that are too thick are difficult to destain, and when counterstained, may obscure acid-fast bacilli.

2. Use contrasting counterstain.
 - a. -Counterstain should not be so intense that it “hides” mycobacteria.
 - b. Color-blind individuals may find fluorochrome-stained smears easier to examine.
3. Beware of false-positive smears. Common sources of acid-fast bacilli which can contribute to this problem are:
 - a. Tap water and infrequently cleaned distilled water reservoirs. Check these sources from time to time to see if acid-fast bacilli can be detected.
 - b. Ice-making machines; ice from these machines is often used to facilitate passage of gastric tubes and may cause false-positive smears or cultures.
 - c. Transfer of material from slide to slide in bulk-staining tanks. To avoid this, stain slides individually.
 - d. Transfer of “positive” flakes from thick slides to other slides via immersion oil. Do not make thick smears; carefully heat-fix smears; wipe oil immersion lens between slides; allow oil to free-fall from applicator onto the smear instead of touching applicator to the slide.

For a more detailed look at acid-fast microscopy, see *Laboratory Manual for Acid- Fast Microscopy* (19).

1. Staining Procedures

Acid-fast staining procedures are of two general types: those employing basic fuchsin dyes and those utilizing fluorochrome dyes. Both types are described below.

BASIC FUCHSIN ACID-FAST STAINS

- a. Ziehl-Neelsen (ZN) Acid-Fast Stain (5,10,19,22). THIS IS A HOT STAIN.

(1) MATERIALS

Basic fuchsin
Ethanol, 95%
Phenol crystals
Hydrochloric acid, concentrated
Methylene blue chloride
Water, distilled

(2) PREPARATION

- (a) **Fuchsin:** Dissolve 0.3 g basic fuchsin in 10 ml 95% ethanol.
- (b) **Phenol:** Weigh 5.0 g phenol crystals; melt with gentle heat; add 100 ml water.
- (c) **Mix solution (a) with 90 ml solution (b); this is now called carbol fuchsin.**
- (d) **Acid alcohol:** Carefully add 3 ml concentrated hydrochloric acid to 97 ml 95% ethanol and gently mix.
- (e) **Methylene blue:** Dissolve 0.3 g methylene blue chloride in 100 ml distilled water.

(3) PROCEDURE

- (a) Prepare smear by spreading material over an area 1 x 2 cm on the slide and allow to air dry.
- (b) Fix smear on an electric slide warmer at 65° to 75°C for at least 2 hours, or use a Bunsen burner flame as for other bacteriological smears. **DO NOT OVERHEAT.**
- (c) Cover the heat-fixed smear with a small (2 x 3 cm) rectangular piece of filter paper. This holds the stain on the slide and minimizes the deposition of dye crystals onto the slide.
- (d) Apply enough carbol fuchsin to the preparation to cover the paper strip.
- (e) Heat the slide to steaming with a Bunsen burner flame or an electric staining rack as follows:
 - (i) With a Bunsen burner flame, gently heat the bottom of the slide until the stain begins to steam. Let stand for 5 minutes without additional heat. Do not boil or allow to dry; add more stain if necessary, but do not reheat.
 - (ii) With an electric staining rack, place slides on the rack, turn switch onto "high," and flood slides with staining solution. When stain begins to steam, let it steam 5 minutes. If necessary, add more stain to prevent slide from drying.

- (f) Remove filter paper strips with forceps and place in discard container. Wash slides with water.
- (g) Flood smear with acid alcohol and destain for 2 minutes.
- (h) Wash smear with water and drain.
- (i) Flood smear with methylene blue and counter-stain for 1 to 2 minutes.
- (j) Rinse with water, drain, and air dry. DO NOT BLOT.
- (k) Examine smear under oil immersion (see 3., pp. 71-75 and 84-85).

b. Kinyoun Acid-Fast Stain (10,13,15,19,22). THIS IS A COLD STAIN.

(1) MATERIALS

Basic fuchsin
Ethanol, 95%
Phenol crystals
Hydrochloric acid, concentrated
Methylene blue
Water, distilled

(2) PREPARATION

- (a) Basic fuchsin: Dissolve 4 g basic fuchsin in 20 ml 95% ethanol.
- (b) Phenol: Dissolve 8 g phenol crystals in 100 ml distilled water (if necessary, heat gently to get into solution).
- (c) Mix solutions (a) and (b). This solution is called **carbol fuchsin**.
- (d) Acid alcohol: Carefully add 3 ml concentrated hydrochloric acid to 97 ml of 95% ethanol, and gently mix.
- (e) Methylene blue: Dissolve 0.3 g methylene blue chloride in 100 ml distilled water.

(3) PROCEDURE

- (a) Prepare smear, by spreading material over 1- x 2-cm area of slide and allow to air dry.

- (b) Fix smear on an electric slide warmer at 65° to 75°C for at least 2 hours or use a Bunsen burner flame as for other bacteriological smears. DO NOT OVERHEAT.
- (c) Cover the heat-fixed smear with a small (2 x 3 cm) rectangular piece of filter paper. This holds the stain on the slide and filters the stain. This is important because the increased dye concentration may leave artifacts on the smear which may be confused with acid-fast bacilli.
- (d) Apply enough Kinyoun carbol fuchsin to the preparation to cover the paper strip. Allow to stain for 5 minutes. DO NOT HEAT.
- (e) Remove paper with forceps and place into discard container. Rinse slide with water and drain.
- (f) Flood the smear with acid alcohol and destain for 2 minutes.
- (g) Rinse smear with water and drain.
- (h) Flood smear with methylene blue and counterstain for 1 to 2 minutes.
- (i) Rinse, drain, and air dry. DO NOT BLOT.
- (j) Examine smear under oil immersion (see 3., pp. 71-75 and 84-85).

FLUOROCHROME ACID-FAST STAINS

These stains employ dyes that fluoresce when excited by ultraviolet or blue light. Just as the basic fuchsin stains, fluorochrome dyes stain acid-fast organisms, and the bacilli are not destained by acid alcohol. This is true acid-fast staining and should not be confused with fluorescent antibody microscopy. More acid-fast smears are found positive by the fluorochrome method than by the Z-N method (4,6), and some microscopists hold that fluorescence microscopy is superior to light microscopy (16,23) for observing acid-fast stained preparations. Fluorochrome-stained smears may lose their fluorescence with time, therefore they should be observed within 24 hours of staining. The smears should not be stained unless they can be observed within this time (19). If stained smears must be kept overnight before being examined, they should be stored at 5°C to minimize the loss of fluorescence.

a. Auramine 0 Fluorescence Acid-Fast Stain (4,15,19,22).

(1) MATERIALS

Auramine 0
Phenol crystals
Hydrochloric acid, concentrated
Ethanol, 70%
Potassium permanganate
Water, distilled

(2) PREPARATION

- (a) Auramine 0: Dissolve 0.1 g auramine 0 in 10 ml 95% ethanol.
- (b) Dissolve 3 g phenol crystals in 87 ml distilled water.
- (c) Mix solutions (a) and (b).
- (d) Acid alcohol: Carefully add 0.5 ml concentrated hydrochloric acid to 100 ml 70% ethanol.
- (e) Potassium permanganate: Dissolve 0.5 g potassium permanganate (KMnO_4) in 100 ml distilled water.

(3) PROCEDURE

- (a) Prepare smear by spreading material over 1- x 2-cm area of slide and allow to air dry.
- (b) Fix smear on an electric slide warmer at 65" to 75°C for at least 2 hours, or use a Bunsen burner flame, as for other bacteriological smears. DO NOT OVERHEAT.
- (c) Flood smear with auramine 0 solution and allow to stain for 15 minutes, making certain that the staining solution remains on the smear. Do not apply heat to smear. Do not use filter paper strips.
- (d) Rinse smear with water and drain.*
- (e) Flood smear with acid alcohol and destain for 2 minutes.
- (f) Rinse smear and drain.

*If rinse water contains an appreciable amount of chlorine, it may interfere with fluorescence. In this case use deionized or distilled water to rinse.

- (g) Flood smear with potassium permanganate and allow to stand for 2 minutes. Do not allow potassium permanganate to act over 3 or 4 minutes or it may quench the fluorescence of acid-fast bacilli.
- (h) Rinse, drain, and air dry,
- (i) Examine smear as soon as possible after staining (see 3., pp. 76-85).

b. Auramine 0-Rhodamine B Fluorescence Acid-Fast Stain (15,18,19,21).

(1) MATERIALS

Auramine 0	Ethanol, 70%
Rhodamine B	Hydrochloric acid, concentrated
Phenol crystals	Potassium permanganate
Glycerol	Water, distilled

(2) PREPARATION

- (a) Auramine 0-Rhodamine B-Phenol: Dissolve 1.5 g auramine 0, 0.75 g rhodamine B in a solution of 75 ml glycerol, 10 ml of melted phenol crystals, and 50 ml distilled water. Mix well. This staining solution may be clarified by filtering through glass wool.
- (b) Acid alcohol: Carefully add 0.5 ml concentrated hydrochloric acid to 100 ml 70% ethanol.
- (c) Potassium permanganate: Dissolve 0.5 g potassium permanganate ($KMnO_4$) in 100 ml distilled water.

(3) PROCEDURE

- (a) Prepare smear by spreading material over 1- x 2-cm area of slide and allow to dry.
- (b) Fix smear on an electric slide warmer at 65° to 75° C for at least 2 hours, or use a Bunsen burner flame as for other bacteriological smears. DO NOT OVERHEAT.

- (c) Flood smear with auramine 0-rhodamine B-phenol solution and allow to stain for 15 minutes, making certain that the staining solution remains on the smear. Do not apply heat to smear. Do not use filter paper strips.
- (d) Rinse smear with water and drain.*
- (e) Flood smear with acid alcohol and destain for 2 minutes.
- (f) Rinse smear and drain.
- (g) Floodsmear with potassium permanganate and allow to act for 2 minutes. Do not allow the potassium permanganate to act over 3-4 minutes or it will quench the acid-fast fluorescence.
- (h) Rinse, drain, and air dry.
- (i) Examine smear as soon as possible after staining (see 3., pp. 76-85).

c. **Auramine 0-Acridine Orange Fluorescence Acid-Fast Stain (20).**

(1) MATERIALS

Auramine 0	Ethanol, 95%
Acridine orange	Ethanol, 70%
Phenol crystals	Hydrochloric acid, concentrated
Sodium phosphate, anhyd. dibasic	Water, distilled

(2) PREPARATION

- (a) Auramine 0. Dissolve 0.1 g auramine 0 in 10 ml 95% ethanol.
- (b) Dissolve 3 g phenol crystals in 87 ml distilled water.
- (c) Mix solutions (a) and (b).
- (d) Acid alcohol: Carefully add 0.5 ml concentrated hydrochloric acid to 100 ml 70% ethanol.

*If rinse water contains an appreciable amount of chlorine, it may interfere with fluorescence. In this case use deionized or distilled water to rinse smear.

- (e) Acridine orange: Dissolve 0.01 g anhydrous dibasic sodium phosphate (Na_2HPO_4) in 100 ml distilled water, then add and dissolve 0.01 g acridine orange.

(3) PROCEDURE

- (a) Prepare smear by spreading material over 1- x 2-cm area of slide and allow to air dry.
 - (b) Fix smear on an electric slide warmer at 65° to 75°C for at least 2 hours, or use a Bunsen burner flame as for other bacteriological smears. DO NOT OVERHEAT.
 - (c) Flood smear with auramine O solution and allow to stain for 15 minutes. Do not apply heat to smear. Do not use filter paper strip.
 - (d) Rinse smear with water and drain.*
 - (e) Flood smear with acid alcohol and destain for 2 minutes.
 - (f) Rinse smear and drain.
 - (g) Flood smear with acridine orange and counter-stain for 2 minutes.
 - (h) Rinse, drain, and air dry.
 - (i) Examine smears as soon as possible after staining (see 3., pp. 76-85).
2. **Sodium Hypochlorite (Clorox) Method for Smear Without Culture (7, 9, 17, 19).**

This method is useful when a smear examination alone is required and when no biological safety cabinet is available to protect personnel from infection when processing specimens by conventional procedures. Because clorox (or other appropriate hypochlorite solutions) will kill most of the organisms in the specimen, the specimen, after being treated with clorox may be handled safely without a safety cabinet. Clorox is bactericidal for mycobacteria and may cause the bacilli to disintegrate if it is allowed to act longer than 15 minutes, therefore, smears should be prepared, stained, and examined promptly.

*If rinse water contains an appreciable amount of chlorine, it may interfere with fluorescence. In this case use deionized or distilled water to rinse smear.

a. **Materials**

- (1) Clorox or other commercial household bleach containing 5% to 6% sodium hypochlorite (NaOCl).
- (2) Screw cap plastic centrifuge tubes, preferably 50 ml.
- (3) Centrifuge and head to provide 2,000 to 3,000 x g.

b. **Procedure**

- (1) Mix equal volumes of sputum and clorox in a screwcap centrifuge tube.
- (2) Tighten cap and shake the tube to liquefy the specimen. A test-tube mixer may be used.
- (3) Let the mixture stand for 15 minutes at room temperature.
- (4) Add distilled water to the 50 ml mark on the tube.
- (5) Centrifuge the tube(s) at 2,000 to 3,000 x g for 15 minutes.
- (6) Decant the supernatant fluid and retain the sediment.
- (7) Suspend the sediment in several drops of water and prepare the smear by spreading a drop of the resuspended material over an area 1- x 2-cm. Allow to air dry.
- (8) Fix the smear by passing the slide through the blue cone of a Bunsen burner flame about three times.
- (9) Perform acid-fast stain as described in the preceding sections.

3. **Examination of Smears**

Learn to use the microscope properly, because improper handling or operating may cause costly damage. Much of the following information regarding light microscopy and fluorescence microscopy has been adapted with permission from earlier work of Smithwick (19) and Koch (14). For a more detailed study of fluorescence microscopy techniques see Jones et al. (12).

LIGHT MICROSCOPY

Figure 19 is a schematic of a microscope; study it to familiarize yourself with the names of the basic parts of this instrument.

a. General Instructions

- (1) Always read the instruction manual for your microscope before attempting to operate it.
- (2) When the microscope is not in use, protect it from dust by a covering or case.
- (3) Always carry a microscope with both hands, one firmly grasping the arm and the other supporting the base.

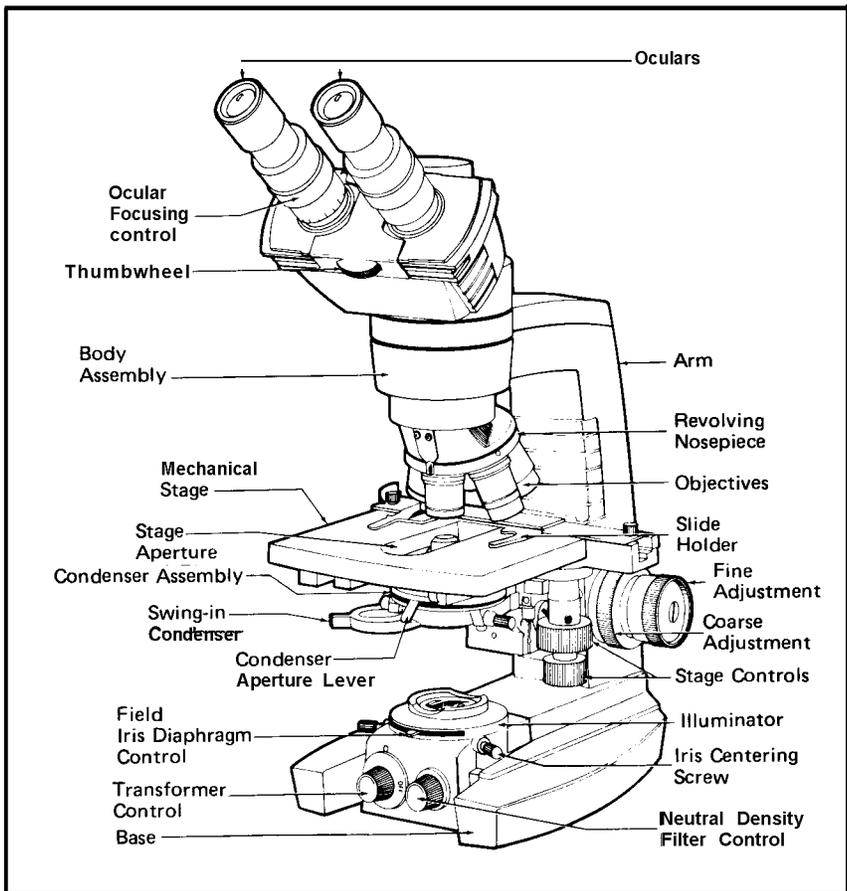


Figure 19. Schematic of a microscope.

- (4) Microscope lenses may be scratched by dirt or grit; therefore, clean the lenses only with **lens** tissue. Never use soap, alcohols, xylene, or other solvents. If lens tissue does not clean the lenses, consult the microscope manufacturer for cleaning recommendations.
- (5) The oil immersion objective lenses should be cleaned after each day's work. Only nondrying, low viscosity, synthetic immersion oils should be used (they are easier to remove).
- (6) Some microscopes have a single eyepiece (monocular); others have two eyepieces (binocular). Most microscopists recommend keeping both eyes open while looking into a monocular microscope with one eye. This prevents fatigue from holding the other eye closed. Although it may be difficult to learn to do this, the effort is worthwhile.

b. Methods of Microscopy

- (1) Microscopes may be used in many ways. Some of these methods will be mentioned to familiarize you with them. A text should be consulted for detailed descriptions.
- (2) In incident light-microscopy the object is lighted from above (epiillumination), and the surface of the object is the most prominent feature.
- (3) In transmitted light microscopy the light passes through the object; variations in color due to staining and differences in refraction and transparency show the form, structure, and other properties of the object.
 - (a) Bright-field microscopy is the most common form of transmitted light microscopy. The light passes directly through the object and is altered only by the object and any dyes used to color this object.
 - (b) In dark-field microscopy the light is transmitted through the object at an angle from the sides rather than from directly below. The refraction and reflection of the light by the object shows its structure in shades of light and dark against a black background.

c. Procedure for Using a Microscope

- (1) Be sure the light source is working properly.
- (2) Be sure the lenses, mirrors, and other light-conducting surfaces are clean.
- (3) Check for broken or damaged parts.
- (4) Adjust the light, mirror, condenser, and diaphragm so that a strong beam of light is directed toward the objective lens.
- (5) Turn the coarse adjustment knob to move the objective lens away from the stage.
- (6) Rotate the nosepiece so that a low power objective lens (5X or 10X) is directly over the condenser.
- (7) Place the slide on the stage so that the object to be viewed is under an objective lens.
- (8) Look from the side of the stage so that you can see the space between the slide and the objective lens. Slowly turn the coarse adjustment knob to bring the objective lens very near, but not near enough to touch the slide, or until the knob stops turning.
- (9) Adjust the light so that it is bright, but not uncomfortably bright, when you look into the eyepiece. This may be done by changing the intensity of the lamp, changing mirror surfaces, using dark filters, adjusting the diaphragm, or adjusting the condenser. Usually, adjusting the diaphragm is sufficient.
- (10) While looking into the eyepiece, slowly turn the coarse adjustment to separate the objective lens and the stage. The object should come into focus within several turns. If not, go back to step 8.
- (11) Turn the fine adjustment knob until the object is seen most clearly.
- (12) While looking from the side, turn the nosepiece to select a higher power lens. Be sure the lens does not touch the slide. The field of view should be almost in focus and can be brought into focus by turning the fine adjustment knob. The intensity of the light may also need to be adjusted.
- (13) To use the oil immersion lens, turn the nosepiece so that no lens is over the object. Let a drop of oil fall on the slide, but do not touch the slide with the

dropper. Now, turn the nosepiece so that the oil immersion lens comes in contact with the oil. Turn the fine adjustment knob to focus.

- (14) Move the slide slowly and systematically across the stage to observe the object.
- (15) Scan the slide as directed for your work or as needed.
- (16) Report only what you see and not what you think you are supposed to see.
- (17) When you have finished with the slide, raise the objective lens, remove the slide, and clean the oil from the lens.
- (18) When you have finished with the microscope, clean the oil off the lens, place the low power objective lens as if it were to be used, turn off the light source, and cover the microscope or place it in its cabinet.

d. **Reading Fuchsin Stained Smears**

Carbol fuchsin stained acid-fast bacilli appear as red stained organisms; with the methylene blue counterstain, nonacid-fast organisms and the background are stained blue (Fig.20).



Figure 20. Short, long, and clumps of red, fuchsin-phenol stained bacilli with a methylene blue stained background.

FLUORESCENCE MICROSCOPY

The lens system of a fluorescence microscope is the same as, or similar to, that of a standard microscope and, after some adjustments, usually may be used for transmitted light microscopy. Any one of several lighting systems can be used. All of these lighting systems require one of the standard electrical power supplies with little or no fluctuation in voltage:

- (1) The high intensity tungsten filament lamp can be used for most fluorescence acid-fast techniques, but not for the fluorescent antibody technique. This is the least expensive and the easiest system to maintain.
- (2) The halogen lamp system can be used for most fluorescence microscopy methods, including the fluorescent antibody.
- (3) The high pressure mercury vapor lamp system has been the most widely used, but it is costly and requires advanced technical skill to operate.
- (4) Some xenon lamps and carbon arcs have been used, but they have not been popular.

The lamp must have its maximum output in that part of the light spectrum that includes both the desired waveband and the light intensity that will excite the stain to its maximum fluorescence. The position of the lamp can be adjusted within its housing to focus the light source on the stained object. The lamp housing usually contains a reflector and/or a collecting lens to aid in concentrating the light on the object.

Many lamps produce enough heat to damage the glass filters transmitting the light; therefore, a heat absorption filter is placed between the lamp and the wavelength selection filters to protect them.

An exciter filter is chosen to absorb the unwanted red, yellow, and green light and to transmit the waveband of light that excites the stain to fluoresce. These exciter filters may be made of colored glass (usually dark blue), or they may be interference type of light filters; they are placed between the heat absorption filter and the stained object. In some microscopes the exciting light is transmitted down through the body tube and the objective lens to excite the fluorochrome stain in the object (epiillumination). This allows the microscopist to scan objects that would not transmit light well, such as rocks, minerals, or thick specimens. This type of illumination may also be used for fluorescence microscopy of smears and tissue sections.

A mirror is usually used to direct the light into the condenser; the mirror must have a front surface of aluminum or silver if it is to reflect ultraviolet light.

The condenser must be designed to provide maximum illumination of the object directly under the objective lens. It may be an oil immersion, dark-field condenser. Some techniques require only a bright-field, dry condenser that focuses the light source on the object (critical illumination).

Between the objective lens and the eye place a filter which absorbs the light passed by the exciter filter, but allows the light emitted by the fluorescing bacilli to pass. This is the barrier filter. It is usually made of colored glass or colored gelatin that has been laminated between two pieces of plain glass: these filters usually appear yellow or orange. The exciter and barrier filters must be properly matched to block out all light from the lamp and still allow the light from the fluorescing stains to reach the eye.

a. **Illuminating Methods in Fluorescence Microscopy**
(1) TRANSMITTED LIGHT FLUORESCENCE

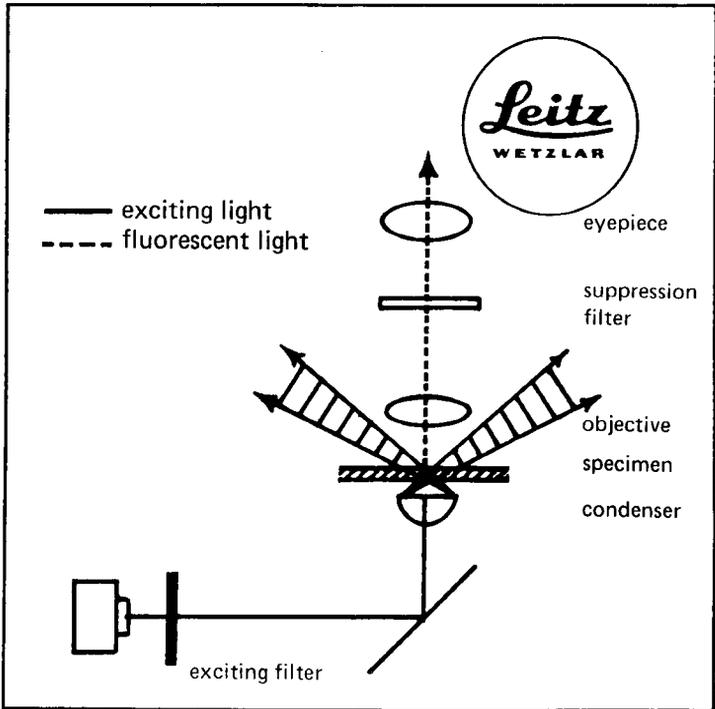


Figure 21. Schematic representation of transmitted light fluorescence system.

Fluorescence excitation by means of transmitted light is the classical method used in fluorescence microscopy today. Either dark-field or bright-field condensers may be used. With dark-field condensers no excitation light reaches the objective, thus insuring a dark background for the specimen. With bright-field condensers, however, excitation light does reach the objective, giving a bright background which reduces the contrast between the background and the bacilli being sought.

(2) INCIDENT LIGHT FLUORESCENCE (EPIILLUMINATION)

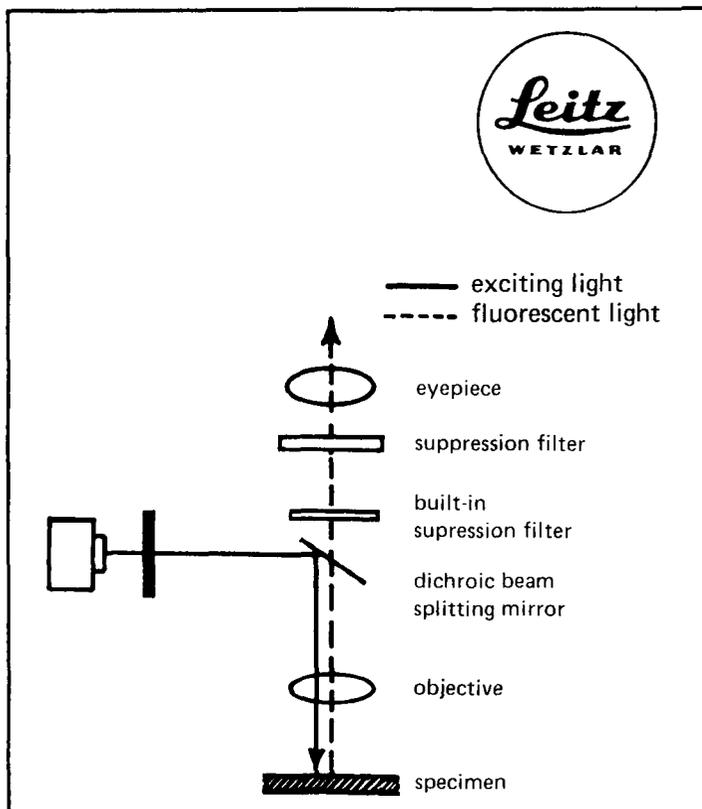


Figure 22. Schematic representation of incident-light fluorescence system.

In fluorescence excitation with incident light (epiillumination), the light is radiated from above the specimen. This insures a strong light intensity, because no light is lost through scattering or absorption in the specimen, a condition possible in transmitted light excitation.

(3) **BLUE LIGHT FLUORESCENCE
ESSENTIAL PARTS OF THE MICROSCOPIC
SYSTEM (See Fig. 23)**

(a) **Light source.**

The conventional light source for the excitation of fluorescent stains has been the ultraviolet light produced by high-pressure mercury vapor bulbs. This is still an excellent source of exciting light, but a cheaper and simpler source is a low-voltage 6V, 15W lamp of high luminous density in a special mount in the base of a microscope. This housing concentrates the light so that sufficient blue rays are collected to excite the auramine O stain.

(b) **Microscope.**

A binocular microscope with bright-field substage condenser is employed. It is cheaper and easier to operate than most other fluorescence equipment, and can also be used to examine smears stained with Ziehl-Neelsen, Gram, etc.

(c) **Lens system.**

- (1) It contains a bright-field substage condenser which does not require the use of oil.
- (2) Planachromat objectives provide a wide, flat field of vision and do not require immersion oil for high magnification.
- (3) Compensating 10X oculars.

(d) **Filters.**

- (1) Exciter filter: 3-mm BG12. The transmission peak is around 404 nm. This is in the visible blue light-ray zone, hence the term "blue-light excitation system."

(2) Barrier filters. These filters will pass the yellow rays emitted by auramine O after it has been excited by blue light.

- (i) Zeiss 50 (or GG 14) transmits rays above 500 nm. The dark green background reveals debris and makes it possible to focus easily on a negative smear. Some workers prefer to use this barrier filter exclusively.
- (ii) Zeiss 53 transmits rays above 530 nm. The background is dark brown, providing the greatest brilliance and contrast. The background debris may be obliterated, which makes focusing difficult on a negative smear.
- (iii) OG-1, a yellow gelatin filter, sometimes used; it transmits above 500 nm.

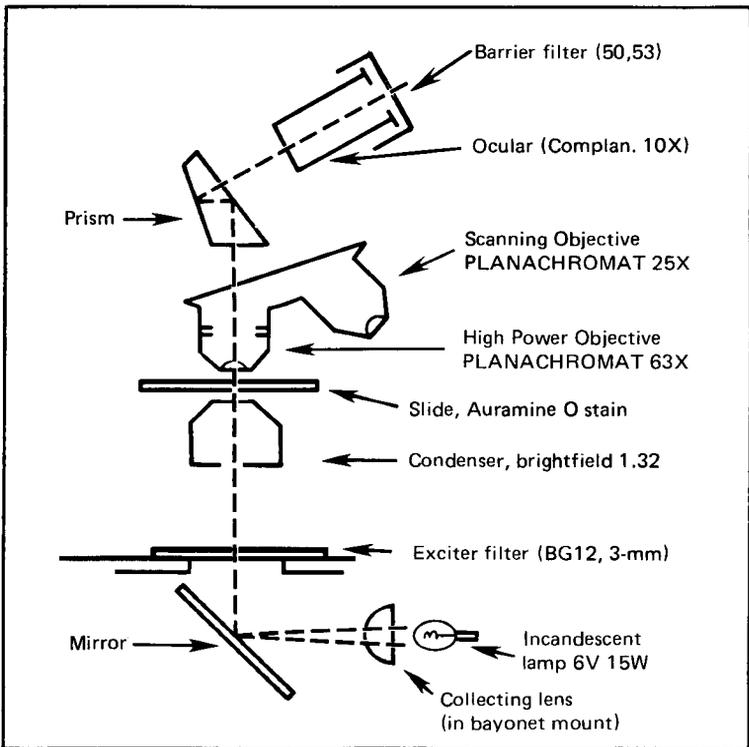


Figure 23. Schematic representation of blue light brightfield microscopy for acid-fast organisms stained with auramine O.

b. **Reading Fluorochrome Stained Smears**

(1) **With High Dry Objectives**

- (a) Examine with fluorescence microscopy equipment outfitted with BG-12 exciter filter and an OG-1 barrier filter.
- (b) With this system auramine O stained organisms emit a bright yellow fluorescence and auramine O-rhodamine B stained organisms emit a yellow-orange fluorescence (Fig.24,25).
- (c) With the potassium permanganate counterstain, the nonspecific fluorescing debris is usually a pale yellow; the acid-fast organisms, bright yellow (Fig.24,25); the background, almost black.
- (d) With the acridine orange counterstain, the background is stained red to orange, which sharply contrasts with the bright yellow acid-fast organisms (Fig.26).
- (e) Smears may be scanned rapidly with a 10X to 25X objective. Occasionally, the 45X or 63X objective may have to be used to ascertain actual bacterial morphology.
- (f) If culture work is not done, or until the microscopist has demonstrated competence, all positive smears should be confirmed with the Ziehl-Neelsen stain.



Figure 24. Yellow fluorescing, auramine O-phenol stained bacilli. The background fluorescence is quenched with potassium permanganate.

- (g) After the fluorochrome smear has been examined, it may be overstained with Z-N without removing the auramine stain. After the smear has been overstained with Z-N, however, it is no longer satisfactory for examination by fluorescence microscopy.



Figure 25. Yellow-orange fluorescing, auramine O-rhodamine B-phenol stained bacilli. The background fluorescence is quenched with potassium permanganate.

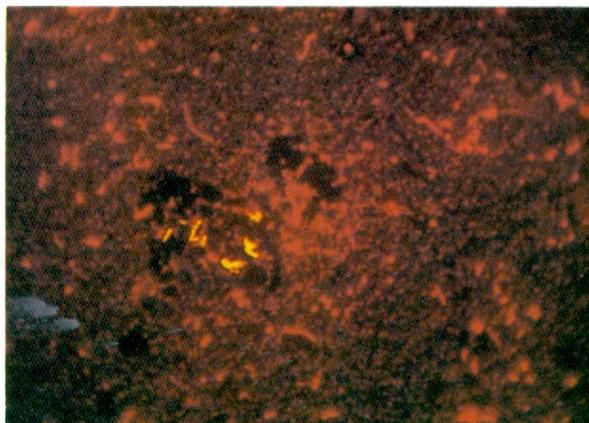


Figure 26. A yellow fluorescing, auramine O-phenol stained bacillus with an orange fluorescing, acridine orange stained background.

(2) With Oil Immersion Objectives

Most acid-fast fluorescence microscopy is performed with low power and high power dry objectives, but some laboratories have old FA equipment outfitted with oil immersion objectives and microscopists use them from time to time. The method follows.

- (a) When fluorescence microscopy equipment has been properly aligned and appropriate filters (BG-12 and OG-1) are in position, examine stained slides as follows.
- (b) Clean objective and condenser lenses carefully with lens paper.
- (c) Lower condenser slightly and place one or two drops of low fluorescence immersion oil (Cargille's type A) on condenser lens.
- (d) Place slide to be examined in mechanical stage and move so that desired area is over condenser lens. Place immersion oil on cover slip.
- (e) Raise condenser slowly until oil contacts slide over an area about 1/2 inch (ca. 12 mm) in diameter. Avoid making bubbles in the oil.
- (f) Examine field with low power (10X) objective and bring into focus.
- (g) Adjust level of condenser (up or down) until light in the microscopic field is concentrated in a small circle.
- (h) Adjust position of circle of light to center of field, using adjusting screws on right and left front area of condenser base.
- (i) When field is in proper focus under low power, and only then, examine with oil-immersion objective.
- (j) Most microscopes are approximately parfocal, but may require slight adjustment on changing from low power to oil immersion objective lens.
- (k) The dark field condenser may also have to be adjusted slightly (up, down, or centered) for the oil immersion objective lens.
- (l) When field is in sharp focus under oil immer-

sion, establish the best possible lighting condition by slightly adjusting the collecting lens and iris diaphragms (if present) in lamp housing assembly and/or oil immersion objective.

- (m) If culture work is not done or until the microscopist feels confident, all positive smears should be confirmed with the Ziehl-Neelsen stain.
- (n) After the fluorochrome smear has been examined, it may be overstained with Z-N without removing the auramine stain. After the smear has been overstained with Z-N, however, it is no longer satisfactory for examination by fluorescence microscopy. Remove residual oil with xylene.

Smear Examination

Careful smear examination is an essential part of today's tuberculosis control program. The microscopist's training should emphasize the importance the clinician attaches to results of smear examination in deciding to discharge a patient to outpatient treatment or to gainful employment (3,8,11).

To obtain excellence in microscopic examination, one should have a good microscope and a comfortable work area. Methods of reading smears vary from laboratory to laboratory. At CDC, no time limit has been set for examining a smear. Instead, a system has been adopted which insures that a representative area of the smear is examined. To insure that an area is covered only once, the smear should be searched in an orderly manner by making a series of 3 horizontal or 9 vertical parallel sweeps of the slide (see Fig.27). All parts of the smear should be sampled. Each field should be searched thoroughly, with a rapid change to the next field. These procedures should be followed regardless of how much time it takes. In this manner, the microscopist should see 60 to 100 fields in one sweep, or as many as 300 fields if he finds it necessary to examine the entire smear. If the smear is moderately or heavily positive, then fewer fields may be examined, and a report of positive may be made even though the entire smear has not been examined.

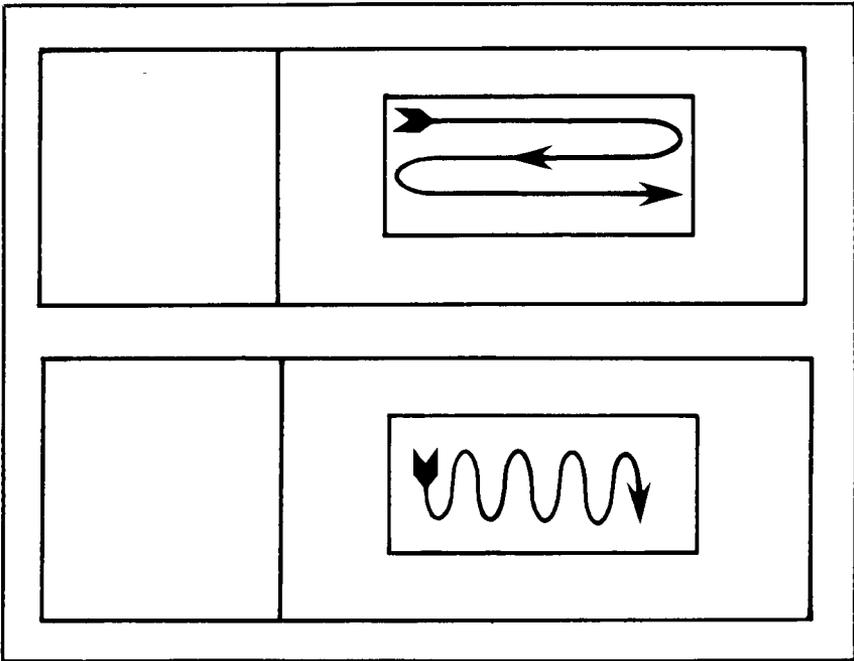


Figure 27. Recommended methods for examining acid-fast stained smears.

Acid-fast bacilli in specimens are usually in the form of rods, but they also may appear coccoid (round-like) or filamentous (long, slender, and even branching). They are frequently bent and may contain heavily stained areas called beads, or alternating stained and clear areas that make them appear to be banded. See Figures 28 and 29. From pure cultures, individual rods of *M. tuberculosis* may be aggregated side by side and end to end to form "cords" (Fig. 30). This is often the case in smears made from the turbid fluid at the bottom of Lowenstein-Jensen slants on which *M. tuberculosis* is growing.

As smears of pure cultures show, not all of the mycobacteria in a given smear are necessarily acid fast. Some species of the genera *Nocardia* and *Corynebacterium* and even some bacterial and fungal spores may be acid fast (2). In fluorescence acid-fast microscopy debris may fluoresce and thus be mistaken for acid-fast bacilli. The microscopist should learn to distinguish acid-fast bacilli from these other sometimes confusing organisms and artifacts by examining smears frequently and thus learning to recognize the morphology of acid-fast organisms.

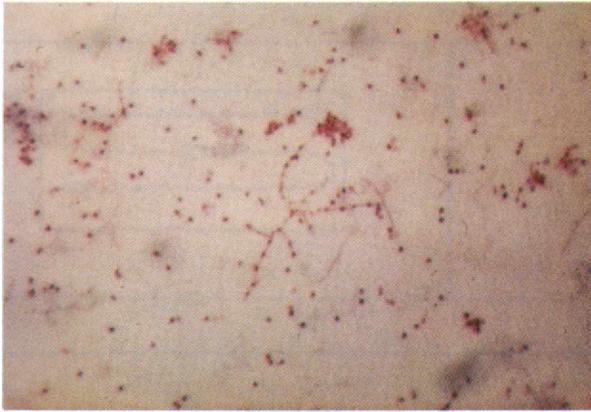


Figure 28. Branching filaments with beading and small coccoid forms. From Smithwick (19).

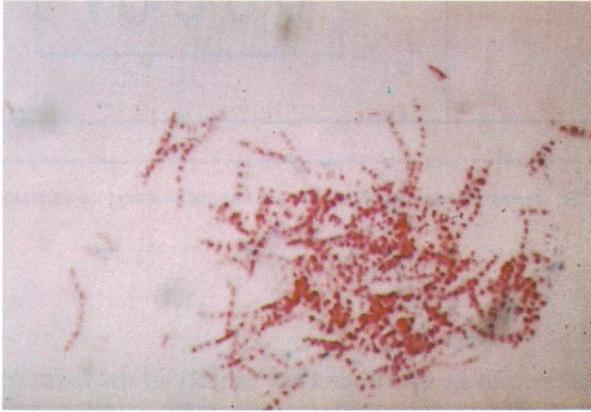


Figure 29. Alternating clear and pink-stained parts of the bacilli that show the characteristic of banding. Note the dark-stained parts called beads. From Smithwick (19).

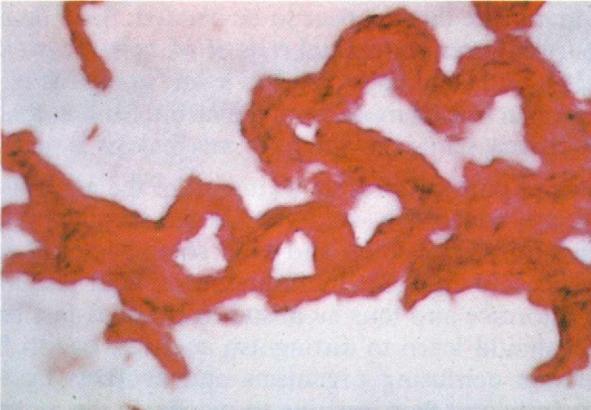


Figure 30. Strands of bacilli in cords. From Smithwick (19).

4. Reporting of Smears

- a. Make sure reports state the staining method used and the number of acid-fast bacilli seen on the smear.
- b. Observe only enough fields to get an average number of acid-fast organisms.
- c. Count a clump of bacilli that are touching as one. Give separate counts to separate organisms. A clump of bacilli is considered a single colony forming unit; this is important to know when determining dilutions of sediment for direct drug susceptibility tests.
- d. Note any large numbers of clumps on the report, thus indicating that the actual number of acid-fast organisms is larger than that actually reported.
- e. **REPORT ONLY THE NUMBER OF ACID-FAST BACILLI SEEN; DO NOT TRY TO "SPECIATE" WITH MICROSCOPY ALONE.**
- f. Counts of less than 3 per 300 fields by fuchsin stains (or 10 per 300 fields by fluorochrome stains) are not considered positive. A report of "doubtful" may be sent or, if the specimen is still available, a second smear may be prepared, stained, and examined. In either case, request another specimen.
- g. Several systems are used for reporting the results of acid-fast microscopy. Two such systems are given here.

(1) Both Smithwick (19) and David (10) recommend the reporting scheme tabulated below (Table 3).

Table 3. Suggested method for reporting the number of acid-fast bacilli observed in smears when scanning at 100X to 1,000X*

Number of AFB found:	Report:	Alternate:
0	Negative for AFB	-
1 - 2 / 300 fields	Number seen**	±
1 - 9 / 100 fields	Number / 100 fields	1+
1 - 9 / 10 fields	Number / 10 fields	2+
1 - 9 / field	Number / field	3+
>9 / field***	>9 / field	4+

*Magnifications less than 800X should be clearly stated on the report or counts should be adjusted as follows: near 650X, divide count by 2; near 450X, divide count by 4; near 250X, divide count by 10. Example: observed average of 8 AFB per 10 fields at 450X, adjusted to 2 AFB per 10 fields at 800X to 1000X.

** Any number of AFB that is less than 3 per 300 fields at 800X to 1000X is not considered positive, but it does indicate that another specimen should be processed if available.

*** > = greater than.

(2) The American Lung Association recommends the following method: (1,22).

Number of Bacilli	Report
0	No acid-fast bacilli found.
1-2 in entire smear	Report number found and request repeat specimen.
3-9 in entire smear	Rare or +
10 or more in entire smear	Few or ++
1 or more per field	Numerous or +++

REFERENCES

1. Ad Hoc Committee to Revise Diagnostic Standards, American Lung Association. 1974. Diagnostic Standards and Classification of Tuberculosis and Other Mycobacterial Diseases. New York, N.Y. 10019. p.31.
2. Barksdale, L., and K. S. Kim. 1977. Mycobacterium. *Bacteriol. Rev.* **41**:217-372.
3. Bates, J. H. 1974. Ambulatory treatment of tuberculosis - An idea whose time has come. *Amer. Rev. Respir. Dis.* **109**:317-319.
4. Bennedsen, J., and S. O. Larsen. 1966. Examination for tubercle bacilli by fluorescence microscopy. *Scand. J. Respir. Dis.* **47**:114-120.
5. Bishop, P. J., and G. Neuman. 1970. The history of the Ziehl-Neelsen stain. *Tubercle.* **51**:196-206.
6. Blair, E. B., O. L. Weiser, and A. H. Tull. 1969. Mycobacteriology laboratory methods, Lab. Report No.323. U.S. Army Medical Research and Nutrition Laboratory, Fitzsimons General Hospital, Denver.
7. Cameron, G. M., and R. Castles. 1946. Detection of tubercle bacilli in sputum: application of the NaOH alum method, the clorox method, and the direct smear. *J. Lab. Clin. Med.* **31**:361-364.
8. Cashman, H. H., N. D. D'Esopo, W. Dickinson, Jr., V. N. Houk, H.G. Muchmore, J. A. Sbarbaro, and E. Wolinsky. 1970. Bacteriologic standards for the discharge of patients. *Amer. Rev. Respir. Dis.* **102**:470-473.
9. Corper, H. J., and C. R. Nelson. 1949. Methods for concentrating acid-fast bacilli. *Amer. J. Clin. Path.* **19**:269-273.
10. David, H. L. 1976. Bacteriology of the mycobacterioses. DHEW Publication No. (CDC) 76-8316. Center for Disease Control, Atlanta, Georgia. Available from Superintendent of Documents, U.S. Government Printing Office, Washington, D. C. 20402.
11. Gunnels, J. J., J. H. Bates, and H. Swindoll. 1974. Infectivity of sputum-positive tuberculosis patients on chemotherapy. *Amer. Rev. Respir. Dis.* **109**:323-330.
12. Jones, G. L., G. A. Hebert, and W. B. Cherry. 1977. Fluorescent antibody techniques and bacterial applications. Center for Disease Control, Atlanta, Georgia 30333.
13. Kinyoun, J. J. 1915. A note on Uhlenhuth's method for sputum examination, for tubercle bacilli. *Amer. J. Pub. Health.* **5**:867-870.
14. Koch, K. F. 1972. Fluorescence microscopy instruments, methods, applications. Ernst Leitz, Wetzlar, Germany.
15. Lennette, E. H., E. H. Spaulding, and J. P. Truant. 1974. Manual of clinical microbiology, 2nd ed. American Society for Microbiology, Washington, D. C.
16. Mitchison, D. A. 1968. Examination of sputum by smear and culture in case-finding. *Bull. Int. Union Against Tuberc.* **41**:139-147.
17. Oliver, J., and T. R. Reusser. 1942. Rapid method for the concentration of tubercle bacilli. *Amer. Rev. Tuberc.* **45**:450-452.

18. Richards, O. W., E. K. Kline, and R. E. Leach. 1941. Demonstration of tubercle bacilli by fluorescence microscopy. *Amer. Rev. Tuberc.* **44**:255-266.
19. Smithwick, R. W. 1976. *Laboratory manual for acid-fast microscopy*. 2nd ed. Center for Disease Control, Atlanta, Georgia 30333.
20. Smithwick, R. W., H. L. David. 1971. Acridine orange as a fluorescent counterstain with the auramine acid-fast stain. *Tubercle.* **52**:226-231.
21. Truant, J. P., W. A. Brett, and W. Thomas, Jr. 1962. Fluorescence microscopy of tubercle bacilli stained with auramine and rhodamine. *Henry Ford Hosp. Med. Bull.* **10**:287-296.
22. Vestal, A. L. 1975. *Procedures for the isolation and identification of mycobacteria*. DHEW Pub. No. (CDC) 75-8230. Center for Disease Control, Atlanta, Georgia 30333.
23. World Health Organization Expert Committee on Tuberculosis. 1974. Ninth annual report. Technical series 552. World Health Organization, Geneva, Switzerland.

E. CULTURE AND PRELIMINARY OBSERVATION OF MYCOBACTERIA ON LOWENSTEIN-JENSEN AND MIDDLEBROOK 7H-10 MEDIUM.

M. tuberculosis is the most common species of *Mycobacterium* found in clinical specimens from patients suspected of having tuberculosis. Other mycobacteria are also found in these specimens, and some may even be associated with disease. Technical personnel in the level II (4) laboratory must therefore be able to identify *M. tuberculosis*, and they must understand that some of these other acid-fast bacilli may be potential pathogens and should be sent to a level III laboratory for precise characterization.

Even though acid-fast microscopy is a valuable aid in today's tuberculosis control programs, it does not provide specific identification of mycobacteria. The organism must be grown on culture medium to permit a definitive diagnosis of *M. tuberculosis*.

Many formulations of media have been proposed for the cultivation of mycobacteria. Most are variations of egg-potato base or serum-agar base formulations. An ideal medium should (i) support rapid and luxuriant growth from minimal inocula, (ii) permit preliminary separation of mycobacteria based upon pigment production and colonial morphology, (iii) inhibit the growth of contaminants, (iv) permit the performance of drug susceptibility tests, (v) be easy to prepare, and (vi) be economical. Unfortunately, no single medium meets all of these criteria, but many of them have been used with varying degrees of success.

Two of the more widely used egg-base formulations are the American Trudeau Society (ATS) medium (7) and Lowenstein-Jensen (L-J) medium (3). Of the agar base formulations, the most popular are Middlebrook 7H-10 medium (5) and 7H-11 medium (1). A general purpose broth medium that can be used for routine diagnostic work (e.g., dilutions, growth studies, and indirect drug susceptibility tests) is Middlebrook 7H-9 broth (5) medium or Dubos- Middlebrook tween-albumin broth (2) medium. Both the agar and broth media are available commercially as powdered bases (with enrichments). The Lowenstein-Jensen medium is available commercially as ready prepared tubed slants and butts. Also available is a commercial powdered base which must be enriched with fresh eggs. A totally self-contained egg base medium (using powdered eggs) has been marketed but has not yet been fully evaluated. The various commercial media work well, but each lot should be checked with a strain of *M. tuberculosis* having known growth characteristics.

Several factors may contribute to the difficulties the microbiologist encounters when trying to culture mycobacteria from digested, decontaminated clinical specimens. Some of these factors are (i) variations in the growth requirements of different strains of mycobacteria, (ii) uneven distribution of mycobacteria in clinical specimens, (iii) harmful effects of the digestant-decontaminant solution on mycobacteria, and (iv) toxicity of some specimen component for the mycobacteria. Because of these factors, digested, decontaminated specimens should be inoculated, both undiluted and after a tenfold dilution (to dilute possible toxic materials in the specimen), onto multiple units of at least two different kinds of basal medium. If space and money permit, at least four units of an egg base medium and one biplate of an agar base medium should be used, as described in III.B.1.c, p. 46. All cultures should be incubated as outlined in III.C, p. 58.

1. Examination of Cultures

When reading cultures, use some type of magnification to better visualize the colonies; a hand lens or dissecting microscope is suitable. With the aid of magnification, the microbiologist can observe young colonies at an early stage of growth, can report detailed morphology of mature colonies, and can more easily detect transparent colony forms.

Cultures should be examined 5 to 7 days after primary inoculation, and at least weekly thereafter for 6 to 8 weeks before they are discarded as negative. If facilities permit, prolonged incubation may be advisable in selected cases. If cultures become contaminated (obvious overgrowth, or, in

the case of LJ medium, when slants become deep blue or pale yellow) this should be reported immediately and another specimen should be requested from that patient.

2. Reporting Observations

a. **Growth rate.** This is defined as the number of days of incubation required for colonies to become visible without the aid of magnification. Rapid growers usually mature within 7 days. Slow growers require more than 7 days. Because the true "speed of growth" of a culture may be grossly exaggerated on primary isolation from clinical material, the true growth rate should be determined only on fresh subcultures.

b. **Amounts of growth.** Record as follows:

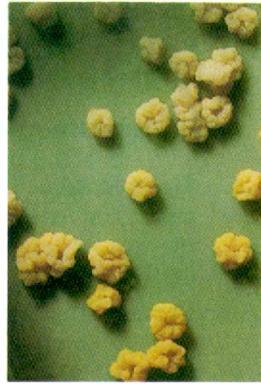
No colonies	= Negative	200-500 colonies = 3+
<50 colonies	= Actual Count	(almost confluent)
50-100 colonies	= 1+	Over 500 colonies = 4+
100-200 colonies	= 2+	(confluent)

c. **Pigment production.** The mycobacteria may be divided into three groups on the basis of pigment production. Pigment formation is best observed in cultures which have isolated colonies. Yellow, orange, or red colonies are considered pigmented or chromogenic (CH). Pale yellow, pink or tan colonies are recorded as such. White, cream, or buff colonies are considered nonpigmented or nonchromogenic (NC). To demonstrate pigment production, cultures should be grown initially in the dark, and later the young, actively growing colonies may be exposed to light. The three groups are identified as:

- (1) **Photochromogens.** cultures whose colonies are nonpigmented when grown in the dark, but become pigmented after they are exposed to light for 1 or more hours and reincubated overnight (Fig. 31).
- (2) **Scotochromogens.** Cultures whose colonies produce pigment when grown either in the dark or the light (Fig. 32). Cultures grown in continuous or prolonged exposure to light may reveal more intense pigment than companion cultures protected from light.
- (3) **Nonphotochromogens.** Cultures whose colonies are nonpigmented or possess only pale shades of pigment, and whose color is unaffected by exposure to light (Fig. 33).



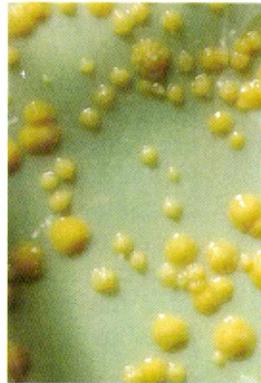
a. Smooth colonies grown in dark on LJ medium.



b. Rough colonies grown on LJ medium; some colonies shielded from light.

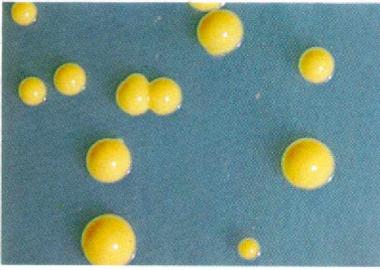


c. Smooth colonies grown on 7H-10 agar.



d. Smooth (some rough) colonies grown on LJ medium.

Figure 31. Colonies of several species of photochromogenic mycobacteria.



a. Smooth colonies on 7H-10 agar



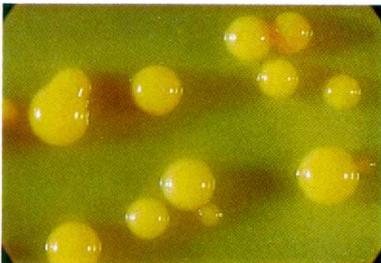
b. Rough colonies on 7H-10 agar



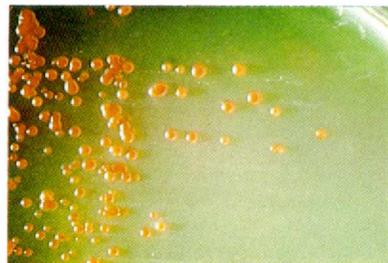
c. Smooth colonies on LJ medium



d. Smooth, pale yellow colonies on 7H-10 agar



e. Smooth colonies on LJ medium

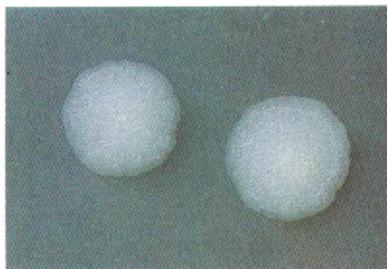


f. Smooth colonies on LJ medium

Figure 32. Colonies of several species of scotochromogenic mycobacteria.



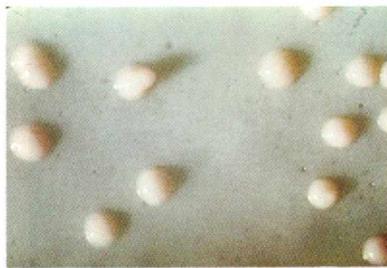
a. Rough colonies on 7H-10 agar.



b. Rough colonies on 7H-10 agar.



c. Smooth colonies on 7H-10 medium.



d. Smooth colonies on egg medium.



e. Smooth colonies on egg medium.



f. Smooth colonies on egg medium.

Figure 33. Colonies of several species of nonphotochromogenic mycobacteria.

3. Colony Morphology

On culture media inoculated with bacterial suspensions diluted to yield isolated colonies, individual colony types may be observed, and described, and they may prove to be helpful in identifying the cultured organism. Some of the descriptive terms for mycobacterial colonies on culture media are listed below. Other symbols for recording colony characteristics can be found in Vestal's manual (6).

a. Surface:

Smooth (Sm)
Rough (R or Rg)
Cording

b. Elevation and Colony Form:

Flat	Umbonate (raised center)
Convex	Rosette (lobate)
Pyramidal	Doughnut

c. Edge:

Entire
Irregular
Flat and Spreading

d. Opacity:

Transparent Opaque
Translucent

e. Pigment (in dark):

Nonpigmented (buff)
Pigmented

“Greening”: Some cultures absorb the malachite green dye from LJ slants during incubation, or when they are stored in the refrigerator.

References

1. Cohn, M. L., R. F. Waggoner and J. K. McClatchy. 1968. The 7H-11 medium for the cultivation of mycobacteria. *Amer. Rev. Respir. Dis* **98**:295-296.
2. Dubos, R. J., and G. Middlebrook. 1947. Media for tubercle bacilli. *Amer. Rev. Tuberc.* **56**:334-345.
3. Holm, J., and V. Lester. 1941. Diagnostic demonstration of tubercle bacilli. *Act. Tuberc. Scand.* *16*:Fasc. 3-4. In *Pub. Hlth. Rep.* **62**:847-854, 1947.

4. Kubica, G. P., W. M. Gross, J. E. Hawkins, H. M. Sommers, A. L. Vestal and L. G. Wayne. 1975. Laboratory services for mycobacterial diseases. *Amer. Rev. Respir. Dis.* **112**:773-787.
5. Middlebrook, G., and M. L. Cohn. 1958. Bacteriology of tuberculosis: laboratory methods. *Amer. J. Pub. Hlth.* **48**:844-853.
6. Vestal, A. L. 1975. Procedures for the isolation and identification of mycobacteria, DHEW Publication No. (CDC) 75-8230. Center for Disease Control, Atlanta, Georgia 30333.
7. Woodruff, C. E., D. Crombie, J. G. Wooley, E. Medlar and W. Steenken. 1946. American Trudeau Society: Committee on evaluation of laboratory procedures. *Amer. Rev. Tuberc.* **54**:428-432.

IV. SPECIFIC IDENTIFICATION OF
MYCOBACTERIUM TUBERCULOSIS

IV. SPECIFIC IDENTIFICATION OF *Mycobacterium tuberculosis*

The procedures presented here are those used at the Centers for Disease Control (CDC). They are not the only methods available, but they have proved to be reliable at CDC and in other public health laboratories for the identification of *M. tuberculosis*.

After being isolated in pure culture, mycobacteria may be grouped roughly by (1) the time and temperature of incubation needed to obtain macroscopic growth—that is, mature colonies on culture media, and (2) the presence or absence of pigment. If the cultures are examined weekly, and their purity has been confirmed by smear examination, most of these observations may be made on primary isolation.

M. tuberculosis is the species most commonly isolated from suspected pulmonary tuberculosis. The appearance of slow-growing, rough colonies on culture media and the detection of niacin in culture extracts is a presumptive identification of this species, but the mycobacteriologist must still perform additional tests to arrive at a definitive identification.

Each culture isolate should be transferred to a liquid medium and then subcultured to solid medium to determine rate of growth and pigment production and to perform the niacin, nitrate reduction, and 68°C catalase tests.

Microscopically, individual cells of *M. tuberculosis* are rod shaped, 2 to 5 μm or more long, and 0.4 to 0.6 μm wide. After dividing, the multiplying cells tend to form tightly packed cords that appear to pile up at the center, giving the colonies a characteristic rough, corded appearance. The colonies are nonpigmented, grow best between 35° and 37°C, and usually require 2 weeks or more to form macroscopic colonies. Although the features are characteristic and suggestive of *M. tuberculosis*, they are not unique to that species. By adding to these features the niacin, nitrate reduction, and 68°C catalase tests, the Level II laboratory should be able to identify *M. tuberculosis* with a high degree of reliability. Recommended methods for performing these tests are outlined below.

A. SPEED OF GROWTH

On primary isolation, even rapidly growing mycobacteria may take 3 weeks or more to appear on the culture medium (8); consequently, the rate of growth should always be determined by using as inoculum

a fresh, liquid subculture diluted sufficiently to yield isolated colonies on whatever solid medium (usually egg-base) is inoculated.

The liquid medium most commonly used is enriched Middlebrook 7H-9 broth (made from commercial ingredients). Once the purity of the unknown culture growing on primary isolation medium has been determined by smear examination, several colonies are aseptically picked and inoculated into 5 ml of 7H-9 broth contained in 20-x 150-mm screwcapped test tubes (or 3 ml of 7H-9 in 16-x 125-mm tubes). Cultures are incubated at 35° to 37°C for 5 to 7 days; shaking the cultures daily by hand will increase the amount of growth at the end of the incubation period (be certain the screwcap is securely tightened before shaking the culture).

At the end of the incubation period, the bacterial suspension is diluted to the "high dilution" (see chart below), on the basis of the extent of turbidity of the broth culture, and 0.1 ml of this dilution is inoculated onto each of several tubes of egg medium. As a safeguard against overdilution, several tubes of media should also be inoculated with the next lower or "medium dilution." The recommended dilutions are given in the chart. The suspension should be diluted as described earlier in I.F.6., p. 27.

Broth Consistency	Dilutions to be made		
	Low	Medium	High
Very turbid	10 ⁻⁴	10 ⁻⁵	10 ⁻⁶
Moderately turbid	10 ⁻² or 10 ⁻³	10 ⁻³ or 10 ⁻⁴	10 ⁻⁴ or 10 ⁻⁵
Slightly turbid	undil. or 10 ⁻¹	10 ⁻¹ or 10 ⁻²	10 ⁻² or 10 ⁻³

Inoculated media are incubated at 35° to 37°C (lower or higher temperatures may be used if other species are expected). Examine tubes after 5 to 7 days of incubation and weekly thereafter.

M. tuberculosis is grouped with the slow-growing mycobacteria because it takes more than 7 days of incubation for mature colonies to be visible to the unaided eye. Tubercle bacilli also exhibit a restricted growth temperature range, growing best between 35° and 39°C; growth of diluted inocula (described above) will not be seen in tubes incubated at 22° to 25°C or at 42°C or above.

B. COLONY MORPHOLOGY

Colonies of *M. tuberculosis* on most egg media are rough, dry, friable, and nonpigmented. They are usually heaped up in the center and have a thin, almost transparent, trailing edge. Occasionally, the col-

onies are flat, rough, and spreading, with only a slight central elevation. Combinations of colony forms between these extremes may be seen. See Figure 34 for examples.

On 7H-10 or 7H-11 agar based media, *M. tuberculosis* grows as flat, rough, spreading, nonpigmented colonies, with only slight elevations in the center. When viewed through a dissecting microscope, by transmitted light, the colonies appear dark, rough, and ropey or corded (because of the close alignment of multiplying bacilli in side-by-side and end-to-end arrangement). See Figure 35 for examples.

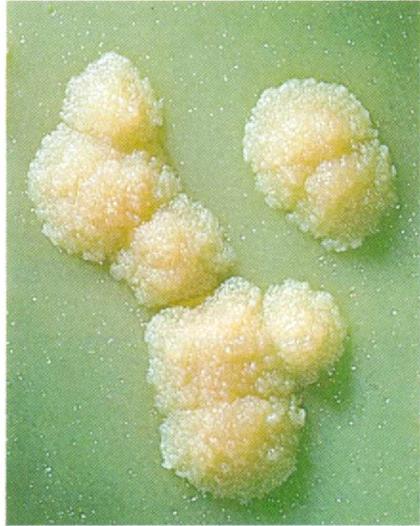


Figure 34. Colonies of *M. tuberculosis* on egg medium.

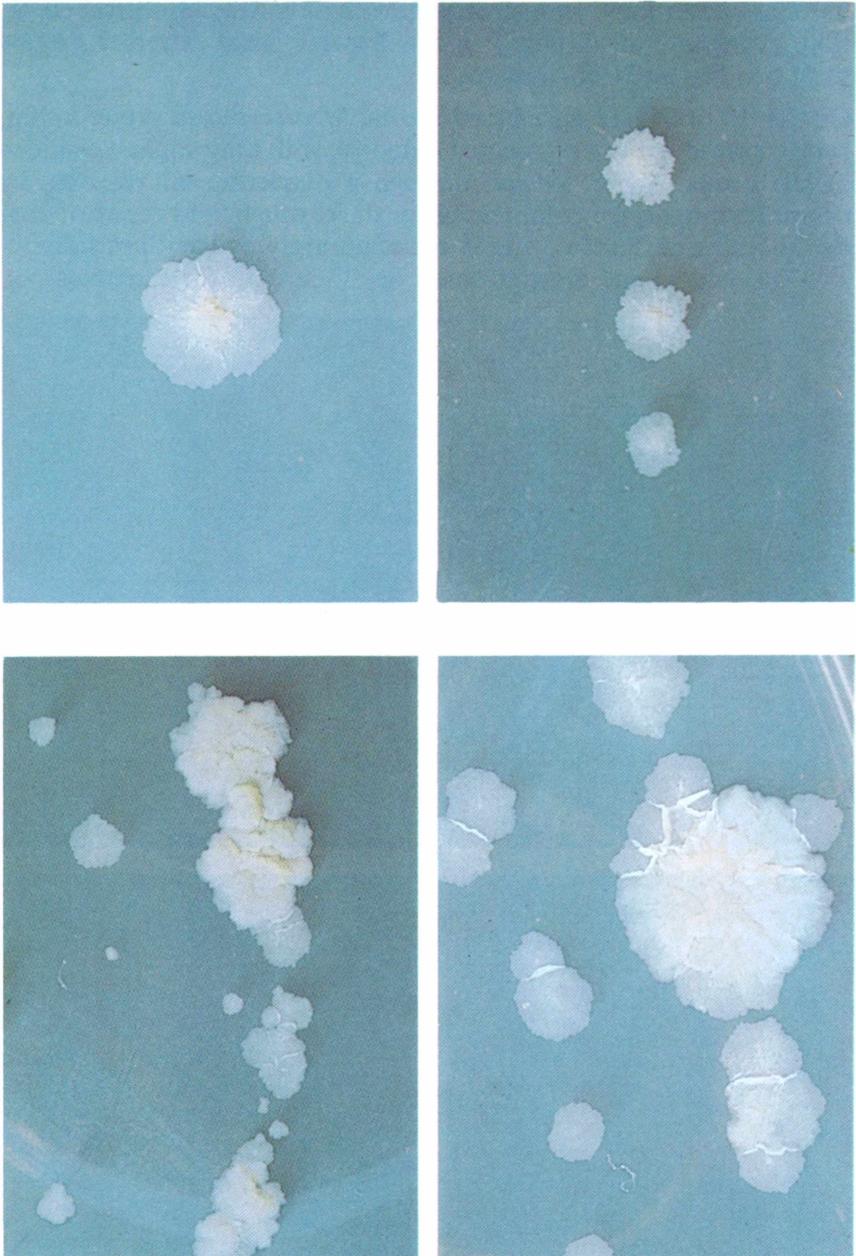


Figure 35. Colonies of *M. tuberculosis* on 7H-10 agar medium.

The microbiologist should also know what *M. tuberculosis* **does not look like** on these two types of media. Although colonies of tubercle bacilli may, on rare occasions, absorb the dye (especially from egg medium) and appear green, they do not appear bright yellow and orange as do some other mycobacteria. Colonies of *M. tuberculosis* are always rough, never smooth and hemispherical (if a tube of medium has an excessive amount of water in it, this may flow over the colonies and make them "appear" smooth, but if the caps are loosened and the moisture dries up, the colonies again look rough). Examples of some of these non-*M. tuberculosis* colonies are shown in Figures 31-33.

C. NIACIN TESTS (6)

This is one of the most reliable in vitro tests for the identification of *M. tuberculosis*. The fact that a few species other than *M. tuberculosis* occasionally produce positive results in the niacin test emphasizes the importance of performing supportive tests to confirm the niacin test reaction.

Most consistent results in the niacin test are obtained from cultures grown on egg medium; therefore, except in unusual circumstances, niacin tests should be performed only on cultures grown on egg-base medium. Cultures grown on 7H-10 or 7H-11 agar media may give negative results unless the media are supplemented with 0.1% potassium aspartate (4) or unless the niacin extraction is carried out at elevated temperature, and usually for a prolonged time (10).

Cultures should be pure (check them with a fuchsin acid-fast stain), have a least 50 or more colonies on the medium, and be 3 to 4 weeks old (if tests are still negative on 4-week-old cultures and cultures have been handled aseptically, reincubate until they are 6 weeks old and retest; otherwise use a fresh subculture). Because the bacteria excrete niacin into the growth medium, a slant with confluent growth may give a negative niacin test result because the extracting fluid cannot contact the culture medium. In such cases, use a transfer spade or the tip of a pipette to scrape away or puncture the growth, thus exposing the surface of the medium. Additional precautions and comments about false positive or false negative niacin test reactions can be found elsewhere (8).

The niacin test may be done either with chemical reagents prepared in the laboratory or with commercially available paper strips for detecting the niacin. Several investigators (2,3,5,14) have obtained comparable results with both procedures. They are described below.

1. Niacin Test With Chemical Reagents (12)

a. **Materials**

4% aniline

10% cyanogen bromide

Sterile distilled water or 0.85% saline

Sterile screwcap test tubes, 16 x 125 mm

95% ethanol

b. **Preparations**

4% aniline solution

To 96.0 ml of 95% ethanol, add 4.0 ml of colorless aniline. Store in a brown bottle in the refrigerator. If solution turns yellow, discard.

10% cyanogen bromide*

Dissolve 5.0 g cyanogen bromide in 50 ml distilled water. Store in a tightly capped brown bottle in the refrigerator. If a precipitate forms upon cooling, warm to room temperature to dissolve. Cyanogen bromide is volatile. Weak solutions give false negative results, so prepare the reagent in small amounts.

0.85% saline

Dissolve 0.85 g sodium chloride in 100 ml distilled water. Sterilize by autoclaving.

c. **Procedures**

- (1) To the culture slant on egg base medium, add 1.0 ml of sterile distilled water or saline.
- (2) Place the tube so that the fluid covers the entire surface of the medium. Allow the extraction to proceed for 15 minutes. Longer extraction times may be used under some circumstances (e.g., cultures with few colonies or cultures known to be weak niacin producers).
- (3) Remove 0.5 ml of the fluid extract and transfer to a clean 16-x 125-mm screwcap tube.
- (4) Add 0.5 ml of the aniline solution.

*Cyanogen bromide is a severe lacrimator, is very toxic, and, in acid solutions, it forms hydrocyanic acid. The compound should, therefore, be handled only in a well-ventilated safety cabinet. The test tubes used in performing the tests should be discarded into a disinfectant solution made alkaline by the addition of sodium hydroxide.

- (5) Add 0.5 ml of the cyanogen bromide solution.
- (6) Observe for the formation of a yellow color, which should develop immediately.

d. **Results**

Positive = yellow color.

Negative = no color change.

See Figure 36.

e. **Controls**

Reagent controls on extract from uninoculated medium (negative).

Negative control on extract from known culture of *M. avium complex*. Positive control on extract of a known culture of *M. tuberculosis*.

2. Paper Strip Procedure (5,14).

A paper strip method for detecting niacin obviates the need to prepare and store unstable chemicals used to demonstrate niacin. Originally described by Kilburn and Kubica (5), these paper strips are now available from several commercial suppliers (Difco, General Diagnostics).

Comparative studies conducted by several researchers (2,3,14, and the authors' personal experience) indicate that these paper strips are as sensitive for detecting niacin production by *M. tuberculosis* as the chemical method described above.

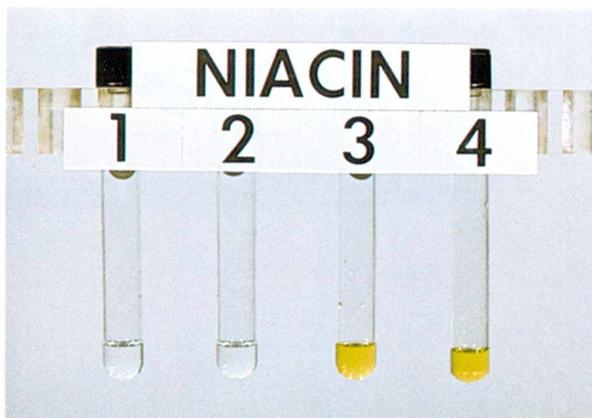


Figure 36. Niacin test with chemical reagents. Tubes 1 and 2 negative; tubes 3 and 4 positive.

a. **Materials**

Reagent-impregnated paper strips

Sterile sealable test tubes (screwcap, cork or rubber stopper): 12 x 75 mm for Difco strips; 13 x 100 mm for General Diagnostics strips.

b. **Procedure**

- (1) Into 0.6 ml of the culture extract, obtained as described under C.1.c., p. 102, insert the strip with identification end up (some strips have an arrow indicating which end of the strip to insert first). Immediately seal the tube.
- (2) Allow the test system to react at room temperature for 15 to 20 minutes; agitate occasionally. Do not tilt the tube.
- (3) Observe the color of the liquid in the **bottom of the tube** against a white background.

c. **Results**

positive = development of yellow color in extract
negative = no color development in extract.

Any color development on the strip itself should be disregarded.

d. **Controls**

Same as indicated for the chemical procedure.

See Figure 37 for examples.

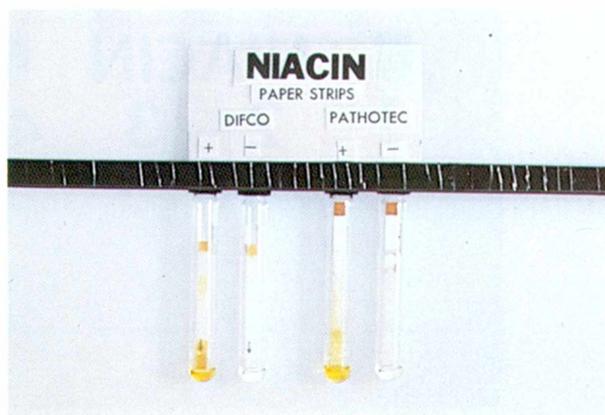


Figure 37. Niacin test by the paper strip procedure.

NOTE: Neutralize the strips with 10% sodium hydroxide before discarding, or discard them into alkaline disinfectant.

D. NITRATE REDUCTION TESTS

The ability of mycobacteria to reduce nitrates (NO_3) to nitrite (NO_2) provides a differential tool that aids in species identification. The test can be performed by using chemical reagents, or reagent-impregnated paper strips or it can be combined with a test for niacin detection. *M. tuberculosis* is one of the strongest reducers of nitrate among the mycobacteria, a trait that makes the paper strip test especially valuable for this species. Other mycobacteria may give results in the paper strip test which conflict with those obtained by the classical procedure with chemical reagents. If reliable results are to be expected for the classical and strip tests, a heavy suspension of the organisms must be used. A light suspension may yield a questionable reaction. The combined test, performed directly on egg-base slants, often gives more intense reactions and more reliable results (personal experience at CDC). Each of the three tests is described here.

1. Nitrate Reduction Test With Chemical Reagents (13).

a. Materials

Screwcap test tubes, 16 x 125 mm
Sterile pipettes, capillary or 1.0 ml, and 5.0 ml
Inoculating spade or applicator sticks
Water bath at 37°C or constant temperature block heater
 NaNO_3 , KH_2PO_4 , $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$,
HCl, Sulfanilamide, N-naphthylethylenediamine
dihydrochloride
Distilled water, zinc powder

b. Preparation

(1) Substrate. 0.01M sodium nitrate in 0.022 M phosphate buffer, pH 7.0

Dissolve the following chemicals in order in 100 ml distilled water:

NaNO_3 -----0.085g

KH_2PO_4 -----0.117g

$\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$ ---0.485g

Sterilize by autoclaving.

- (2) Reagent #1. **Carefully** add 50.0 ml concentrated HCl to 50.0ml distilled water (**NEVER ADD WATER TO ACID**).
- (3) Reagent #2. Dissolve 0.2g sulfanilamide in 100.0 ml distilled water.
- (4) Reagent #3. Dissolve 0.1 g N-naphthylethylenediamine dihydrochloride in 100.0 ml distilled water. Store the substrate and reagents in the dark under refrigeration. If a precipitate forms or the reagents change color, discard and prepare afresh.

c. **Procedure**

- (1) Use a 3 to 4 week old culture from Lowenstein-Jensen or some other egg base medium.
- (2) Add 0.2 ml sterile distilled water to a sterile 16-x 125-mm screwcap test tube.
- (3) Using a sterile spade or applicator stick, emulsify 2 spadesful of growth into the water.
- (4) Add 2.0 ml of the NaNO₃ substrate solution. Shake by hand and incubate in upright position in 37°C water bath for 2 hours. If water bath is not available, a pan of water may be placed in a 37°C incubator, and test cultures placed in this pan for incubation. Heat transfer in the water "medium" is more rapid and insures better enzymic activity.
- (5) Remove from the temperature bath.
- (6) Add one drop of reagent #1.
- (7) Add two drops of reagent #2.
- (8) Add two drops of reagent #3.

d. **Results**

Positive = Red color development, which may range from pale pink (±) to deep red (5+) by comparison with the color standards described in the appendix. *M. tuberculosis* generally gives a 3+ to 5+ color reaction.

Negative = No color development. If no color develops, the test is either negative or the reduction has proceeded beyond nitrite. To confirm, add a small amount of powdered zinc to the mixture. The zinc catalytically reduces nitrate to nitrite, and therefore a true negative

test becomes red when zinc is added. If there is no color change when zinc dust is added to an apparently negative reaction tube, the test may have been positive with reduction having gone beyond nitrite. In such cases, repeat the test. See Figure 38 for examples.

Controls

Positive = *M. tuberculosis*, which is usually a 3+ to 5+.

Negative = Reagents without organisms.

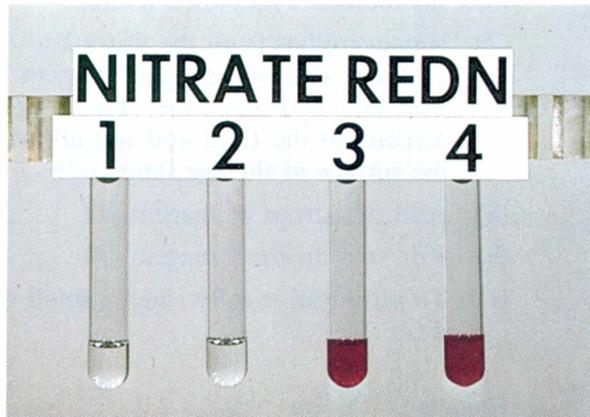


Figure 38. Nitrate reduction test with chemical reagents. Tubes 1 and 2 negative; tubes 3 and 4 positive.

2. Combined Niacin/Nitrate Test (7).

a. **Materials**

Same as in D.1.a., p. 105.

b. **Preparation**

Same as in D.1.b., p. 105.

c. **Procedure**

- (1) Use a 3-to 4-week old culture from Lowenstein-Jensen or some other egg base medium.
- (2) Add 2 ml of the nitrate substrate to each slant.

- (3) Place the tube so that the fluid covers the slants and let stand for 15 minutes*.
- (4) If necessary, remove 0.6 ml of the nitrate solution and place in a clean tube for the niacin test (use correct tube size, depending upon niacin test method employed).
- (5) With the tip of a pipette, dislodge several colonies into the remaining solution.
- (6) Incubate at 37°C for 2 hours.
- (7) Remove tubes from the water bath and acidify the solution with one drop of reagent #1. Be certain that this and the other two reagents are added directly to the fluid and not allowed to run down the surface of the egg slant.
- (8) Add two drops of reagent #2
- (9) Add two drops of reagent #3
- (10) To all negative tubes, add a small quantity of zinc dust.

d. **Results**

- (1) Make observations as described in the standard nitrate test (D.1., p. 105).
- (2) The niacin test may be performed and read by any of the methods described earlier.

See Figure 39 for examples of the reaction.

3. Nitrite Test Strips for Detection of Nitrate Reduction (1,11).

a. **Materials**

Reagent-impregnated test strips (Difco, General Diagnostics)

*An alternative method used at CDC is to add 2 ml of the buffered nitrate substrate to the test culture and to place the tube **upright** in a 37°C water bath for 2 hours (NOTE: if the substrate solution is not in direct contact with some of the colonies on the slant when the tube is upright, dislodge some growth into the substrate with the pipette tip.) **After** the 2 hour incubation, remove 0.5 to 0.6 ml of the substrate solution to a clean tube for the niacin test (using any desired procedure), and add reagents for the nitrate reduction test as described above. In contrast to the usual niacin test procedure (C.1.c., p. 102), the prolonged time for extraction of niacin and the higher temperature of 37°C used here often makes positive niacin test reactions more definitive.

Sterile screwcap test tubes, 13 x 100 mm

Sterile spade or applicator sticks

Forceps

Water bath, 37°C or constant-temperature block heater.

Sterile saline, 0.85%

b. **Procedure**

- (1) Use a 3 to 4 week old culture from Lowenstein-Jensen or some other egg base medium.
- (2) Add 1.0 ml sterile saline to a sterile screwcap 13-x 100-mm test tube.
- (3) Using a sterile spade or applicator stick, remove two spadesful of growth from the culture and add to the saline; break up the culture with the spade or stick.
- (4) Using flamed forceps, carefully insert a nitrite test strip so the bottom of the strip is inserted first. Hold the tube in a vertical position and do not let the strip contact any fluid on the sides of the tube. Cap tightly and incubate at 37°C for 2 hours.
- (5) Shake tube gently at end of first and second hour of incubation. Do not tilt the tube at this point.
- (6) After 2 hours of incubation, carefully tilt the tube back and forth six times to wet the entire strip.
- (7) Slant tube at room temperature to cover the strip with the liquid. Let it remain in this position for 10 minutes.

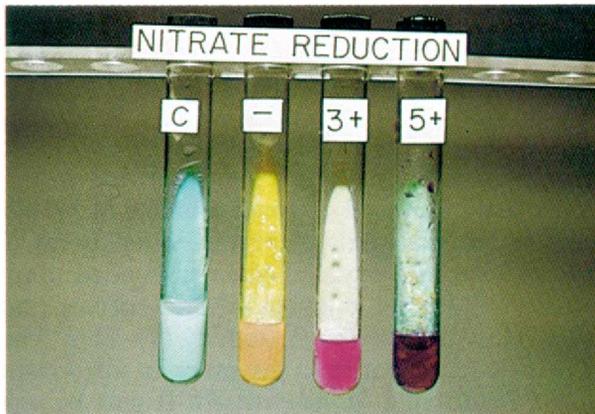


Figure 39. Nitrate reduction test by the combined method.

d. **Results**

Positive = Top portion of strip changing to light blue or dark blue (Difco); central portion of strip changing to pink (General Diagnostics).

Negative = No color change.

See Figure 40 for examples.

The test strips are sensitive to excessive heat, light, and moisture. Store between 2° to 8°C in the original container, tightly capped. Any discoloration on the strips indicates that the reagent has deteriorated and the strips should be discarded.

e. **CAUTION:** see page 112.

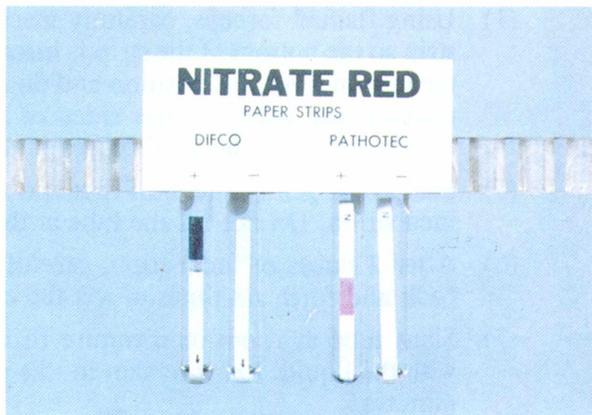


Figure 40. Nitrate reduction test by the paperstrip procedure.

E. CATALASE TEST AT pH 7/68°C (9)

Except for some isoniazid-resistant mutants of *M. tuberculosis*, most mycobacteria possess catalase enzymes which break down hydrogen peroxide to water and oxygen. When suspended in pH 7 buffer and heated to 68°C for 20 minutes, several species (most notably *M. tuberculosis*) lose this enzymatic activity (9). This “hot catalase” test, then, is a valuable adjunct to the other in vitro tests described for specifically identifying *M. tuberculosis*. It may be especially helpful with strains of tubercle bacilli that give negative or only weakly positive results in niacin tests.

1. Materials

Pipettes, 1.0 ml

Sterile screwcap test tubes, 16 x 125 mm

Water bath or constant temperature block heater, 68°C

Inoculating spade or loop

30% hydrogen peroxide (Superoxol). Store in refrigerator

10% Tween 80

0.067 M Phosphate buffer, pH 7

2. Preparation

a. **10% Tween 80.** Mix 10.0 ml Tween 80 with 90.0 ml distilled water. Autoclave for 10 minutes at 121°C. Store in the refrigerator. Immediately before performing the test, mix equal parts of the Superoxol and the Tween 80 solution (0.5 to 1.0 ml will be needed for each test).

b. **0.067 M phosphate buffer, pH 7.0** (see III.B.1.b. (2) (a), p. 45).
Mix 61.1 ml of disodium phosphate with 38.9 ml of monopotassium phosphate. Check pH on meter or with pH test paper.

3. Procedure

- a. Use a culture from solid media.
- b. With a sterile pipette, dispense 0.5 ml of the pH 7 phosphate buffer into a sterile 16-x 125-mm screwcap test tube.
- c. With a sterile spade or applicator stick, emulsify one spadeful of growth from the culture into the buffer.
- d. Place the tubes at 68°C for 20 minutes (the time and temperature are critical).
- e. Remove from heat and cool to room temperature.
- f. Add 0.5 ml of the Tween-peroxide mixture.

4. Results

Observe for the formation of bubbles at the surface of the liquid. Hold the tubes for 20 minutes before reporting as negative.

Positive = Bubble formation

Negative = No bubble formation

See Figure 41 for examples.

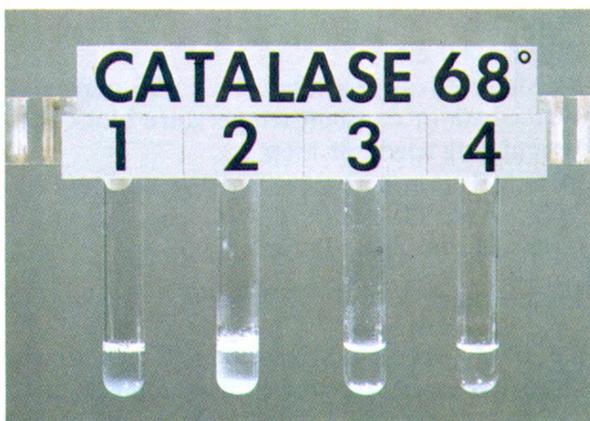


Figure 41. The pH7/68° C catalase test.
Tubes 1 and 2 positive; tubes 3 and 4 negative.

Controls

Positive = *M. fortuitum*

Negative = *M. tuberculosis* or *M. gastri*

WARNING - 30% hydrogen peroxide is a strong OXIDANT. Handle with care; rubber gloves are recommended.

Using the three in vitro tests described (niacin, nitrate reduction, and pH 7/68°C catalase tests), CDC personnel have demonstrated a high degree of reliability for the tests in the specific identification of *M. tuberculosis*. Of more than 200 strains of *M. tuberculosis* examined as unknowns, 96% were observed to yield the correct reactions in these three tests, i.e., positive results in niacin and nitrate reduction tests and negative results in pH 7/68°C catalase tests. Of more than 1,000 other unknown mycobacteria studied, less than 1 in 1,000 reacted in in vitro tests like *M. tuberculosis*.

The paper strip method for detecting niacin production has been approved by most who have used it, and it appears especially valuable for laboratories that have trouble maintaining fresh chemical reagents. The nitrite test strip is less reliable for some species of mycobacteria that are weak reducers of nitrate. Most strong nitrate reducers, notably *M. tuberculosis*, give generally acceptable reactions in the paper strip test for nitrate reduction, and at this time we recommend the strip test for this species of *Mycobacterium* only.

CAUTION: If control cultures of *M. tuberculosis* fail to give consistently good results in any laboratory, the paper strip test for nitrate reduction should be abandoned.

REFERENCES

1. Difco laboratories. Bulletin no. 3183.
2. DiSalvo, A. F., and G. N. Lindler. 1970. Evaluation of a paper strip test for detection of niacin production by mycobacteria. *Amer. J. Clin. Pathol.* **53**:871-873.
3. Gangadharam, P. R. J., and A. J. Droubi. 1971. A comparison of four different methods for testing the production of niacin by mycobacteria. *Amer. Rev. Respir. Dis.* **104**:434-437.
4. Kilburn, J. O., K. D. Stottmeier, and G. P. Kubica. 1968. Aspartic acid as a precursor for niacin synthesis by tubercle bacilli grown on 7H-10 agar medium. *Amer. J. Clin. Pathol.* **50**:582-586.
5. Kilburn, J. O., and G. P. Kubica. 1968. Reagent-impregnated paper strips for detection of niacin. *Amer. J. Clin. Pathol.* **50**:530-532.
6. Konno, K. 1956. New chemical method to differentiate human-type tubercle bacilli from other mycobacteria. *Science* **124**:985.
7. Kubica, G. P. 1964. A combined niacin-nitrate reduction test for use in the identification of mycobacteria. *Acta. Tuberc. Pneumol. Scand.* **45**:161-167.
8. Kubica, G. P., W. M. Gross, J. E. Hawkins, H. M. Sommers, A. L. Vestal and L. G. Wayne, 1975. Laboratory services for mycobacterial diseases. *Amer. Rev. Respir. Dis.* **112**:773-787.
9. Kubica, G. P., and G. L. Pool. 1960. Studies on the catalase activity of acid-fast bacilli. I. An attempt to subgroup these organisms on the basis of their catalase activities at different temperatures and pH. *Amer. Rev. Respir. Dis.* **81**:387-391.
10. Neimeister, R. P. 1969. A preliminary report on the technique of extracting niacin from *Mycobacterium tuberculosis* cultured on 7H-10 agar. *Amer. Rev. Respir. Dis.* **100**:401-402.
11. Quigley, H. J., and H. R. Elston. 1970. Nitrite test strips for detection of nitrate reduction by mycobacteria. *Amer. J. Clin. Pathol.* **53**:663-665.
12. Runyon, E. H., M. J. Selin and H. W. Harris. 1959. Distinguishing mycobacteria by the niacin test. *Amer. Rev. Tuberc.* **79**:663-665.
13. Virtanen, S. 1960. A study of nitrate reduction by mycobacteria. *Acta. Tuberc. Scand. Suppl.* **48**:1-119.
14. Young, W. D., Jr., A. Maslansky, M. S. Lefar and D. P. Kronish. 1970. Development of a paper strip test for detection of niacin produced by mycobacteria. *Appl. Microbiol.* **10**:939-945.



V. DRUG SUSCEPTIBILITY TESTS

V. DRUG SUSCEPTIBILITY TESTS

Drug susceptibility tests for tubercle bacilli are among the most difficult procedures to standardize. Several standard methods have been proposed (1) and are in use throughout the world. The method most widely used in the United States, and the one to be discussed later, is designed to determine the proportion of bacilli in the test population which is resistant to the drug(s) being tested.

Because most cases of newly diagnosed tuberculosis in previously untreated patients in the United States are caused by drug susceptible tubercle bacilli (i.e., primary drug resistant cases are not common), most clinicians prescribe antituberculosis chemotherapy without the benefit of drug susceptibility tests. In those areas where primary resistance is recognized as a problem, however, susceptibility tests should be done **before therapy is started**. Unless there is a clinical reason to suspect the emergence of resistant bacilli (as outlined immediately below), susceptibility tests need only be run on the first set of positive specimens, preferably before initiation of treatment. For certain patients susceptibility tests should be required. These groups include (1) patients who suffer relapses, (2) patients whose sputa become culture positive after having been negative, (3) patients whose sputa remain positive after 3 to 6 months of aggressive chemotherapy, (4) patients whose secretions show a definite and persistent increase in detectable acid-fast bacilli after the numbers of such bacilli previously had been declining, and (5) patients suspected of being infected with drug resistant bacilli.

Proficiency in performance of susceptibility tests for tubercle bacilli demands an understanding of (1) the origin of drug resistance, (2) the effect of medium components on the minimal inhibitory concentrations (MIC) of the drugs tested, (3) the stability of the drugs both during storage and in a medium, (4) the type of susceptibility test performed, (5) the criteria of resistance, and (6) the reading and reporting of test results. These points are thoroughly discussed in several publications (1-4,8).

The submerged disc method of susceptibility testing described here and recommended (9,10) for use in Level II laboratories was first described in 1966 (11). The procedure has been critically evaluated (5-7) and has been shown to obviate many of the problems associated with susceptibility testing, such as drug instability, imprecision in weighing and diluting drugs, mislabeling of media, and the difficulty of preparing quality media.

A. PREPARATION OF MEDIUM

1. Enriched Middlebrook 7H-10 agar medium, made according to directions from commercially available powdered base and oleic acid-albumin-dextrose-catalase (OADC) enrichment, is recommended for susceptibility testing of tubercle bacilli. Prepare only the volume needed to test those cultures immediately at hand.
2. Directions for preparing most commonly needed volumes of medium are summarized in Table 4.

Table 4. Preparation of enriched Middlebrook 7H-10 agar from commercially available ingredients,* 1-5.

Final volume needed (ml)	Wt. of 7H-10 powdered basal medium needed (in g)	Vol. of water (ml)	Reagent grade glycerol (ml)		Add OADC enrichment (ml)	Total number of quadrants (not plates) to be prepared
50	0.95	45	0.25	Swirl to suspend.	5	9-10
100	1.9	90	0.5	Autoclave at 121°C	10	18-20
200	3.8	180	1.0	for 10 to 15 mins.	20	38-40
500	9.5	450	2.5	Cool to 50°-52°C	50	98-100
1000	19.0	900	5.0		100	198-200

- * 1. When calculating number of quadrants or plates needed, do not forget that duplicate sets of plates are needed for each culture because two different inocula are used in the test.
 2. Do not forget to include the drug-free control quadrants in your calculated volume of medium needed.
 3. **NOTE: Experience has shown that preboiling the basal medium before autoclaving results in a medium of inferior quality. Avoid this by swirling the powdered base into suspension just before autoclaving.**
 4. Do not allow sterile medium base, with or without oleic acid-albumin-dextrose-catalase (OADC) enrichment added, to stand in 50° to 52°C water bath too long. Temper medium to 50°C, add OADC, and pour medium into plates within 1 hour.
 5. Protect prepared medium from exposure to daylight coming through laboratory windows.
3. The paper drug-containing discs currently available are listed in Table 5. Not all antituberculosis drugs are available in disc form; if special requests are made for tests of susceptibility to drugs not listed in Table 5, the laboratory must either prepare its own medium or refer the request to a reference laboratory where such tests are done (e.g., state health department, or even CDC, provided the request is routed through the State laboratory).

Table 5. Drug-containing discs for susceptibility tests.*

DRUG	** Mcg of Drug in Disc	Final mcg/ml Drug in Medium
Isoniazid	1	0.2
	5	1.0
Streptomycin	10	2.0
	50	10.0
Rifampin	5	1.0
	25	5.0
Ethambutol	25	5.0
	50	10.0
p-Aminosalicylic Acid	10	2.0
	50	10.0
Ethionamide	25	5.0
Kanamycin	30	6.0

* Do not forget to include drug-free control quadrants in your susceptibility test.

Remember: Duplicate sets of drug plates are needed for each test culture because two different inocula must be used.

** Discs are coded to identify both the drug and its concentration.

4. Appropriate drug discs are dispensed aseptically into the center of individual quadrants of sterile plastic dishes. This may be done while the basal medium is being sterilized in the autoclave or tempered at 50°C before the OADC enrichment is added.
5. Exactly 5.0 ml of sterile, tempered (50°C), complete 7H-10 medium is pipetted over the disc. Remember the disc must remain submerged and not be allowed to float to the top. Let the medium solidify. See Figure 42 for example of drug susceptibility test media made by using commercially available drug-impregnated discs.
6. Incubate the plates overnight at room temperature to permit the drug to diffuse uniformly. Discs containing ethambutol lose activity rapidly when allowed to diffuse overnight; after medium has solidified over ethambutol discs, the plates should be placed at 5°C to permit diffusion of drug.

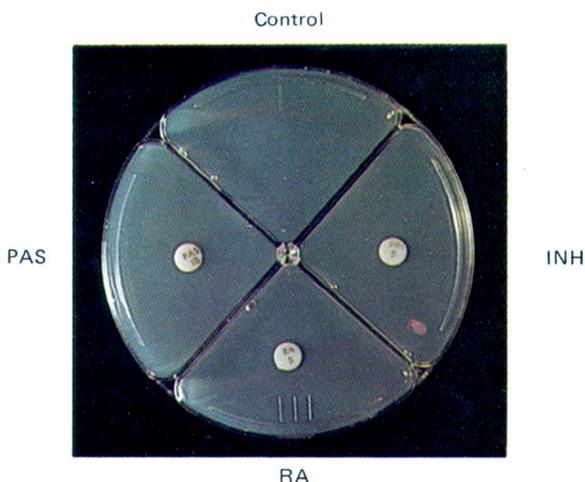


Figure 42. Drug susceptibility test medium made with drug-impregnated discs.

7. Storage of Drug Medium

Since 7H-10 agar medium in plates dehydrates more readily when stored than does egg medium in tubes, the 7H-10 drug medium should be used within 1 month of preparation. This recommendation is for drug medium prepared "from scratch" with powdered drug. Because of the very low final concentrations of most antituberculosis drugs incorporated into media, coupled with the difficulty of weighing and diluting small amounts of drugs or their solutions, most investigators elect to make more-than-needed quantities of drug medium and to store it for future use.

The proper use of drug-impregnated discs insures both a stable and precisely measured quantity of drug. The exact number of drug plates needed for a given day can be prepared without the worries of quantitation and stability; therefore, long-term storage is no longer a problem. This means that a drug medium can be prepared on the day before it is needed and problems of storage, dehydration, or loss of drug activity can be avoided. This alone should greatly improve the quality of drug susceptibility test results.

B. TYPE OF SUSCEPTIBILITY TEST

Either the direct or the indirect drug susceptibility test may be used. The inoculum for the direct test is a digested, decontaminated sputum (or other specimen) in which acid-fast bacilli may be

demonstrated in stained smear. The inoculum size is adjusted on the basis of numbers of bacilli counted in the smear, and the tested bacilli are representative of the bacterial population in the lesion(s) of the host.

The inoculum for the indirect test is a subculture of the organism. Such a test would be needed when (i) smears are negative but cultures positive, (ii) growth on the control medium of a direct test is inadequate (less than 50 colonies), and (iii) a reference culture is submitted for testing. The inoculum for the indirect test is adjusted turbidimetrically.

Details for performing each test are given below.

1. Direct Test

- a. Stain and examine smears of concentrated clinical specimens, and, after first observing an acid-fast organism, record the average number of bacilli observed in the next 20 fields. Count clumps as only one organism, because they will give rise to only one colony on the medium.
- b. Select dilutions of sputum concentrate to be used as inocula on the basis of Ziehl-Neelsen smear results as follows:

Microscopy	Inoculate
Less than 1 AFB/field	Undiluted and 10^{-2}
1-10 AFB/field	10^{-1} and 10^{-3}
More than 10 AFB/field	10^{-2} and 10^{-4}

- c. Make dilutions as described in I.F.6., p. 27.
- d. Use a capillary pipette to inoculate three drops onto each quadrant of the drug and control media. Inoculate one set of plates with the highest dilution and the duplicate plates with the lower dilution.

If the patient has been under treatment, one plate should also be inoculated with undiluted inoculum, regardless of smear results. Bacilli from such patients may be stained, but because of drug action, they may be nonviable.

- e. Include a set of control plates inoculated with a drug-susceptible strain of *M. tuberculosis* (the H37Rv strain is commonly used). This serves as a quality control check on the presence of drug in the discs.
- f. Place plates, medium side down, in individual polyethylene bags and seal.

- g. Incubate at 35° to 37°C in an atmosphere of 10% CO₂.
- h. Read and report results of drug tests at 3 weeks. If cultures are fully matured, and **only if they show drug resistance**, they may be reported in less than 3 weeks. Even though colonies may be fully matured on control media in less than 3 weeks, “drug susceptible” reports should not be tendered until the third week, because resistant colonies often grow more slowly than susceptible ones and may not come up until the third week.
- i. On drug medium colonies appearing after 3 weeks do not necessarily represent resistant bacilli. Some drugs are only bacteriostatic and may be inactivated on prolonged incubation to substatic levels, thereby permitting still fully susceptible colonies to grow **after** 3 weeks. Such colonies are usually smaller than those on the control quadrant.
- j. Drug plates containing the control quadrants should not be discarded as negative until the end of the fifth or sixth week. This is not to permit a reading of the drug susceptibility test, but rather to use the control quadrant as another “tube” of isolation medium, because it has been inoculated with the digested-decontaminated specimen.
- k. Examine all grossly “negative” quadrants with the aid of a dissecting microscope (30x to 60x); drug resistant or very slowly growing mycobacteria may be invisible to the naked eye.
- l. Record the colony characteristics on the control medium. Report results obtained with both dilutions of inoculum and calculate the percent resistance. A convenient way to record growth is as follows:

Confluent (500 or more colonies)	4+
Almost confluent (200-500 colonies)	3+
100-200 colonies	2+
50-100 colonies	1+
Less than 50 colonies	Actual count
- m. For a test to be valid, the control must show good growth (at least 50 to 100 colonies), but not so much as to simulate drug resistance due to growth of spontaneously occurring drug-resistant mutants (i.e., only confluent growth on control). In a properly performed drug susceptibility test one of the control media should have countable colonies so that the percentage of resistant organisms can be

calculated. The only time a control quadrant with confluent growth is acceptable as a valid test is when the culture exhibits total susceptibility to each drug tested.

2. Indirect Test

To prepare inoculum from growth on solid medium:

- a. Scrape several spadesful (2 to 5 mg) of growth from drug-free medium. Try to pick a portion from each colony.
- b. Transfer to a sterile 16-x 125-mm screwcap tube containing 6 to 8 glass or plastic beads* and 3 ml of Tween-albumin liquid medium.
- c. Homogenize on a test tube mixer for 5 to 10 minutes.
- d. Allow larger particles to settle. Withdraw supernatant suspension and adjust density to that of a MacFarland No. 1** with sterile distilled water or saline.
- e. Dilute to 10^{-2} and 10^{-4} (see I.F.6., p. 27 for dilution) in sterile distilled water or saline.
- f. Proceed as for Direct Test for inoculation, incubation, and reading (see V.B.1.d-i. and k.-m., pp. 119-120).

To prepare inoculum with Tween-albumin liquid culture:

- a. Transfer a portion of each colony from drug-free medium into a Tween-albumin liquid medium, such as Middlebrook 7H-9.
- b. Incubate at 35°C for 7 days or until turbidity matches that of MacFarland No. 1 standard.
- c. Dilute to 10^{-3} and 10^{-5} in sterile water or saline.
- d. Inoculate the two dilutions, incubate, and read as described for the Direct Test (V.B.1.d.-m., pp. 119-120).

*Glass beads, 1-2 mm diameter, or high-heat polystyrene plastic beads, such as Styron (R) 700, 27 clear, No. 7, from Dow Chemical Co.

**Prepare MacFarland No. 1 standard by adding 0.1 ml of 1% BaCl_2 to 9.9 ml of 1% H_2SO_4 .

C. Reporting Test Results

1. The report should contain:

The type of test, **Direct** or **Indirect**.

The amount of growth on the control medium.

The amount of growth on each drug medium.

The concentration of each drug in the medium.

A rough calculation of the percentage of bacilli resistant to the drug.

2. This formula may be used to calculate percent:

$$\frac{\text{Number of colonies on the drug}}{\text{Number of colonies on the control}} \times 100 = \% \text{ resistant}$$

Example a.

Drug	Growth on		% Resistant
	Undilute	10 ⁻²	
Control	4+	150C	
1NH 0.2 μg	1+	9C	6
SM 2.0 μg	0	0	0
EMB 5.0 μg	0	0	0

$$\frac{9 \text{ colonies}}{150 \text{ colonies}} \times 100 = 6\%$$

In this example 6% of the organisms were resistant to 0.2 μg/ml INH.

Example b.

Drug	Growth on		% Resistant
	10 ⁻¹	10 ⁻³	
Control	4+	60C	
1NH 0.2 μg	120C	0	2
SM 2.0 μg	0	0	0
RIF 1.0 μg	0	0	0

The 10^{-1} control inoculum gave 4+, or colonies too numerous to count. The hundredfold dilution (10^{-3}) gave 60 countable colonies. Therefore, the 4+ = 60 x 100 or 6000 colonies. Knowing this figure, the proportion of resistant bacilli may be calculated as 2% to 0.2 $\mu\text{g}/\text{ml}$ 1NH.

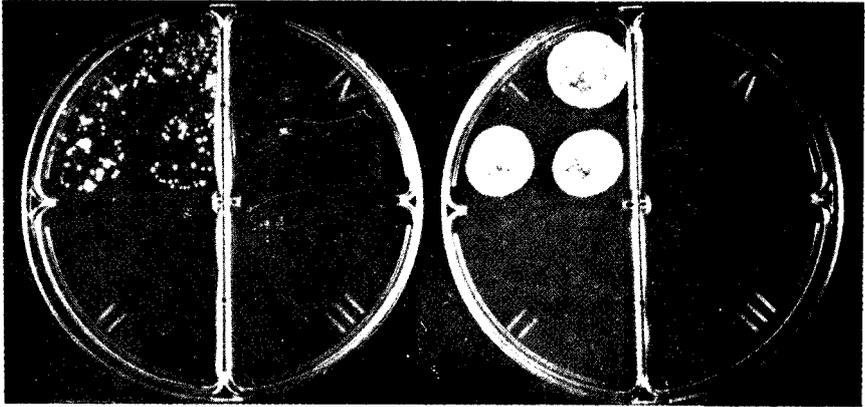
$$\frac{120}{6000} \times 100 = 2\%$$

For other examples, see Figures 43 to 45 on pages 124-126.

If more than 1% of the test population is resistant to the drug under test, this suggests that resistance to that drug has developed or is in an advanced stage of development.

REFERENCES

1. Canetti, G., S. Froman, J. Grosset, P. Hauduroy, M. Langerova, H.T.Mahler, G. Meissner, D. A. Mitchison, and L. Sula. 1963. Mycobacteria: laboratory methods for testing drug sensitivity and resistance. *Bull. Wld. Hlth. Org.* **29**:565-578.
2. Canetti, G., N. Rist, and J. Grosset. 1963. Mesure de la sensibilite du bacille tuberculeux aux drogues antibacillaires par la methode des proportions. *Rev. Tuberc.* **27**:217-272.
3. David, H. L. 1970. Probability distribution of drug-resistant mutants in unselected populations of *Mycobacterium tuberculosis*. *Appl. Microbiol.* **20**:810-814.
4. David, H. L. 1971. Fundamentals of drug susceptibility testing in tuberculosis. DHEW. Pub. No. 00-2165. Center for Disease Control, Atlanta, Georgia 30333.
5. Griffith, M., M. L. Barrett, H. L. Bodily, and R. M. Wood. 1967. Drug susceptibility tests for tuberculosis using drug impregnated discs. *Amer. J. Clin. Path.* **47**:812-817.
6. Griffith, M. E., M. L. Matajack, M. L. Bissett, and R. M. Wood. 1971. Comparative field test of drug-impregnated discs for susceptibility testing of mycobacteria. *Amer. Rev. Respir. Dis.* **103**:423-426.
7. Hawkins, J. E. 1976. Disc and dilution media for susceptibility testing against primary antituberculosis drugs (Abstract). *Amer. Rev. Respir. Dis.* **113/4**(part 2): 78.
8. Kubica, G. P. 1977. Susceptibility testing of tubercle bacilli, In Bondi, A., J. T. Bartola, and J. E. Prier, Eds. *The Clinical Laboratory as an Aid in Chemotherapy of Infectious Disease*, University Park Press, Baltimore, Maryland. pp. 107-114.
9. Kubica, G. P., W. M. Gross, J. E. Hawkins, H. M. Sommers, A. L. Vestal, and L. G. Wayne, 1975. Laboratory services for mycobacterial diseases. *Amer. Rev. Respir. Dis.* **112**:773-787.
10. Vestal, A. L. 1975. Procedures for the Isolation and Identification of Mycobacteria. DHEW Pub. No. (CDC) 75-8230, Center for Disease Control, Atlanta, Georgia 30333.
11. Wayne, L. G. and I. Krasnow. 1966. Preparation of tuberculosis susceptibility testing mediums by means of impregnated discs. *Amer. J. Clin. Path.* **45**:769-771.



INH 0.2

SM 2.0

The organisms inoculated onto these plates were susceptible to 0.2 mcg/ml INH (isoniazid), 2.0 mcg/ml SM (streptomycin), and 2.0 mcg/ml PAS (para-amino-salicylic acid).

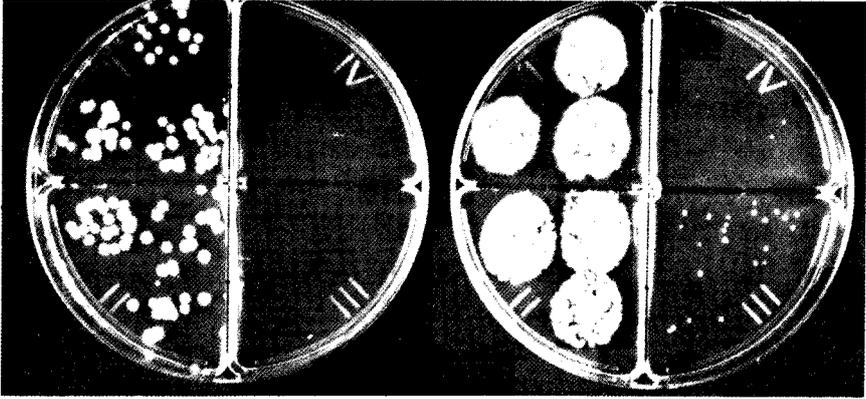
State _____ (Zip Code) _____		<input type="checkbox"/> G <input type="checkbox"/> Ur <input type="checkbox"/> Spinal <input type="checkbox"/> Pleural <input type="checkbox"/> Culture <input type="checkbox"/> First <input type="checkbox"/> Repeat	<input type="checkbox"/> INH <input type="checkbox"/> SM <input type="checkbox"/> PAS <input type="checkbox"/> KM If repeat, date last specimen submitted Mo. _____ Day _____ Year _____																																																							
Laboratory Report EXAMINED MICROSCOPICALLY BY: <input checked="" type="checkbox"/> ZIEHL-NEELSEN <input type="checkbox"/> FLUOROCHROME ACID FAST BACILLI: <input checked="" type="checkbox"/> Found <input type="checkbox"/> Not Found <input type="checkbox"/> Bare <input checked="" type="checkbox"/> Few <input type="checkbox"/> Numerous FUNGI OR YEAST: <input type="checkbox"/> Found <input type="checkbox"/> Not Found <input type="checkbox"/> Split Given to Mycology Lab.	DRUG SUSCEPTIBILITY TESTS IN 7H-10 AGAR <input checked="" type="checkbox"/> DIRECT <input type="checkbox"/> INDIRECT <table border="1" style="width:100%; border-collapse: collapse; text-align: center;"> <thead> <tr> <th>Drug (µg/ml)</th> <th colspan="2">Growth* at Dis.</th> <th>Percent of Pop.</th> </tr> <tr> <td></td> <td>10^{-1}</td> <td>10^{-3}</td> <td></td> </tr> </thead> <tbody> <tr> <td>CONTROL 0.0</td> <td>4+</td> <td>2+</td> <td>100</td> </tr> <tr> <td>INH 0.2</td> <td>0</td> <td>0</td> <td>0</td> </tr> <tr> <td>INH 1.0</td> <td></td> <td></td> <td></td> </tr> <tr> <td>SM 2.0</td> <td>0</td> <td>0</td> <td>0</td> </tr> <tr> <td>SM 10.0</td> <td></td> <td></td> <td></td> </tr> <tr> <td>PAS 2.0</td> <td>0</td> <td>0</td> <td>0</td> </tr> <tr> <td>PAS 10.0</td> <td></td> <td></td> <td></td> </tr> <tr> <td>KM 5.0</td> <td></td> <td></td> <td></td> </tr> <tr> <td>VM 5.0</td> <td></td> <td></td> <td></td> </tr> <tr> <td>THA 5.0</td> <td></td> <td></td> <td></td> </tr> <tr> <td>CS 20.0</td> <td></td> <td></td> <td></td> </tr> <tr> <td>ETH 5.0</td> <td></td> <td></td> <td></td> </tr> </tbody> </table> *4+ = > 500, 3+ = 200 - 500, 2+ = 100 - 200, 1+ = 50 - 100 < 50 recorded & followed by "c", as 10C = 10 colonies	Drug (µg/ml)	Growth* at Dis.		Percent of Pop.		10^{-1}	10^{-3}		CONTROL 0.0	4+	2+	100	INH 0.2	0	0	0	INH 1.0				SM 2.0	0	0	0	SM 10.0				PAS 2.0	0	0	0	PAS 10.0				KM 5.0				VM 5.0				THA 5.0				CS 20.0				ETH 5.0				IDENTIFICATION <input checked="" type="checkbox"/> <i>M. tuberculosis</i> <input type="checkbox"/> <i>M. bovis</i> <input type="checkbox"/> <i>M. kansasii</i> <input type="checkbox"/> <i>M. marinum</i> <input type="checkbox"/> <i>M. scrofulaceum</i> <input type="checkbox"/> <i>M. szulgai</i> <input type="checkbox"/> <i>M. gordonae</i> <input type="checkbox"/> <i>M. flavescens</i> <input type="checkbox"/> <i>M. avium complex (intracellulare)</i> <input type="checkbox"/> <i>M. xenopi</i> <input type="checkbox"/> <i>M. terrae complex</i> <input type="checkbox"/> <i>M. triviale</i> <input type="checkbox"/> <i>M. gastri</i> <input type="checkbox"/> <i>M. fortuitum complex</i> <input type="checkbox"/> Rapid Grower <input type="checkbox"/> Other Mycobacterium
Drug (µg/ml)	Growth* at Dis.		Percent of Pop.																																																							
	10^{-1}	10^{-3}																																																								
CONTROL 0.0	4+	2+	100																																																							
INH 0.2	0	0	0																																																							
INH 1.0																																																										
SM 2.0	0	0	0																																																							
SM 10.0																																																										
PAS 2.0	0	0	0																																																							
PAS 10.0																																																										
KM 5.0																																																										
VM 5.0																																																										
THA 5.0																																																										
CS 20.0																																																										
ETH 5.0																																																										
FINAL REPORT MAY REQUIRE 8 WEEKS SPECIMEN UNSATISFACTORY FOR TESTING BECAUSE: <input type="checkbox"/> Insufficient Amount <input type="checkbox"/> Leaked in Transit <input type="checkbox"/> Not mycobacterium <input type="checkbox"/> Contaminated <input type="checkbox"/>		TENTATIVE IDENTIFICATION: _____																																																								
Date Received and Laboratory Specimen Number: Month _____ Day _____ Year _____ Form No. _____ Specimen Number _____		Date Reported: 1st _____ 2nd _____ 3rd _____ Form Control Number _____																																																								

SAMPLE LABORATORY REPORT FORM

Figure 43. Drug susceptibility test. From Vestal (10).

CON.

PAS 2.0



INH 0.2

SM 2.0

The inoculum (10^{-1}) on the right shows 4+ growth on the controls and INH quadrants, 23 colonies on SM, and 2 on PAS. A 100-fold dilution (10^{-3}) of the inoculum on a second drug plate shows 59 colonies on the control, 54 on INH, and none on SM and PAS. Hence, 54/59 gives 92% resistance to INH. To determine percent resistant to SM and PAS, multiply $59 \times 100 = 5900$ colony units inoculated on to each quadrant of the 10^{-1} plate.

$$23/5900 \times 100 = 0.4\% \text{ R to SM.}$$

$$2/5900 \times 100 = 0.03\% \text{ R to PAS.}$$

Site last
when submitted

State (Zip Code) First Repeat

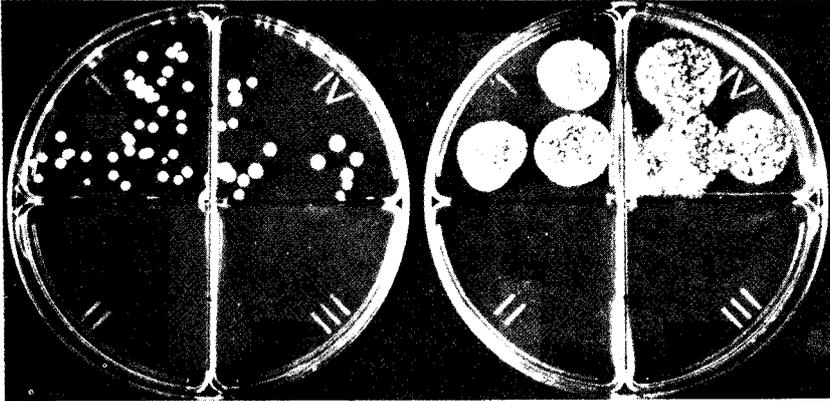
Laboratory Report	DRUG SUSCEPTIBILITY TESTS IN 7H-10 AGAR	IDENTIFICATION																																																								
EXAMINED MICROSCOPICALLY BY: <input checked="" type="checkbox"/> ZIEHL-NEELSEN <input type="checkbox"/> FLUOROCHROME ACID FAST BACILLI: <input type="checkbox"/> Found <input type="checkbox"/> Not Found <input type="checkbox"/> Rare <input checked="" type="checkbox"/> Few <input type="checkbox"/> Numerous FUNGI OR YEAST: <input type="checkbox"/> Found <input type="checkbox"/> Not Found <input type="checkbox"/> Split Given to Mycology Lab.	<input checked="" type="checkbox"/> DIRECT <input type="checkbox"/> INDIRECT <table border="1" style="width: 100%; border-collapse: collapse; text-align: center;"> <thead> <tr> <th>Drug (µg/ml)</th> <th colspan="2">Growth* at Dilts.</th> <th>Percent of Pop.</th> </tr> <tr> <td></td> <th>10^{-1}</th> <th>10^{-3}</th> <td></td> </tr> </thead> <tbody> <tr> <td>CONTROL 0.0</td> <td>4+</td> <td>59c</td> <td>100</td> </tr> <tr> <td>INH 0.2</td> <td>4+</td> <td>54c</td> <td>92</td> </tr> <tr> <td>INH 1.0</td> <td></td> <td></td> <td></td> </tr> <tr> <td>SM 2.0</td> <td>23c</td> <td>0</td> <td>0.4</td> </tr> <tr> <td>SM 10.0</td> <td></td> <td></td> <td></td> </tr> <tr> <td>PAS 2.0</td> <td>2c</td> <td>0</td> <td>0.03</td> </tr> <tr> <td>PAS 10.0</td> <td></td> <td></td> <td></td> </tr> <tr> <td>KM 5.0</td> <td></td> <td></td> <td></td> </tr> <tr> <td>VM 5.0</td> <td></td> <td></td> <td></td> </tr> <tr> <td>THA 5.0</td> <td></td> <td></td> <td></td> </tr> <tr> <td>CS 20.0</td> <td></td> <td></td> <td></td> </tr> <tr> <td>ETH 5.0</td> <td></td> <td></td> <td></td> </tr> </tbody> </table> <p>*4+ = > 500, 3+ = 200 - 500, 2+ = 100 - 200, 1+ = 50 - 100 <50 recorded & followed by "c", as 10C = 10 colonies</p>	Drug (µg/ml)	Growth* at Dilts.		Percent of Pop.		10^{-1}	10^{-3}		CONTROL 0.0	4+	59c	100	INH 0.2	4+	54c	92	INH 1.0				SM 2.0	23c	0	0.4	SM 10.0				PAS 2.0	2c	0	0.03	PAS 10.0				KM 5.0				VM 5.0				THA 5.0				CS 20.0				ETH 5.0				<input type="checkbox"/> <i>M. tuberculosis</i> <input type="checkbox"/> <i>M. bovis</i> <input checked="" type="checkbox"/> <i>M. kansasii</i> <input type="checkbox"/> <i>M. marinum</i> <input type="checkbox"/> <i>M. scrofulaceum</i> <input type="checkbox"/> <i>M. szulgai</i> <input type="checkbox"/> <i>M. goodii</i> <input type="checkbox"/> <i>M. flavescens</i> <input type="checkbox"/> <i>M. avium complex (intracellulare)</i> <input type="checkbox"/> <i>M. xenopi</i> <input type="checkbox"/> <i>M. terrae complex</i> <input type="checkbox"/> <i>M. triviale</i> <input type="checkbox"/> <i>M. gastri</i> <input type="checkbox"/> <i>M. fortuitum complex</i> <input type="checkbox"/> Rapid Grower <input type="checkbox"/> Other Mycobacterium
Drug (µg/ml)	Growth* at Dilts.		Percent of Pop.																																																							
	10^{-1}	10^{-3}																																																								
CONTROL 0.0	4+	59c	100																																																							
INH 0.2	4+	54c	92																																																							
INH 1.0																																																										
SM 2.0	23c	0	0.4																																																							
SM 10.0																																																										
PAS 2.0	2c	0	0.03																																																							
PAS 10.0																																																										
KM 5.0																																																										
VM 5.0																																																										
THA 5.0																																																										
CS 20.0																																																										
ETH 5.0																																																										
FINAL REPORT MAY REQUIRE 8 WEEKS SPECIMEN UNSATISFACTORY FOR TESTING BECAUSE: <input type="checkbox"/> Insufficient Amount <input type="checkbox"/> Leaked in Transit <input type="checkbox"/> Not mycobacterium <input type="checkbox"/> Contaminated <input type="checkbox"/>	TENTATIVE IDENTIFICATION:																																																									
Date Received and Laboratory Specimen Number Month Day Year Form No. Specimen Number	Date Reported 1st 2nd 3rd	Form Control Number																																																								

SAMPLE LABORATORY REPORT FORM

Figure 44. Drug susceptibility test. See Vestal (10).

CON.

PAS 2.0



INH 0.2

SM 2.0

The inoculum on the plate at the right (10^{-1}) is too heavy to determine actual colony counts. In the hundredfold dilution of inoculum (10^{-3}) on the plate to the left we count 41 colonies on the control and 17 on the PAS quadrant. Hence, the apparent resistance to PAS observed in the heavily inoculated plate is a true one, and approximately 41% of the population was resistant to PAS ($17/41 \times 100 = 41\%$).

State (Zip Code)		Culture		Repeat specimen submitted																																																								
		<input type="checkbox"/> First		Mo.	Day	Year																																																						
		<input type="checkbox"/> Repeat																																																										
Laboratory Report		DRUG SUSCEPTIBILITY TESTS IN 7H-10 AGAR			IDENTIFICATION																																																							
EXAMINED MICROSCOPICALLY		<input checked="" type="checkbox"/> DIRECT <input type="checkbox"/> INDIRECT			<input type="checkbox"/> <i>M. tuberculosis</i> <input type="checkbox"/> <i>M. bovis</i> <input type="checkbox"/> <i>M. kansasii</i> <input type="checkbox"/> <i>M. marinum</i> <input checked="" type="checkbox"/> <i>M. scrofulaceum</i> <input type="checkbox"/> <i>M. szulgai</i> <input type="checkbox"/> <i>M. goodii</i> <input type="checkbox"/> <i>M. flourescens</i> <input type="checkbox"/> <i>M. avium complex (intracellulare)</i> <input type="checkbox"/> <i>M. xenopi</i> <input type="checkbox"/> <i>M. terrae complex</i> <input type="checkbox"/> <i>M. triviale</i> <input type="checkbox"/> <i>M. gastri</i> <input type="checkbox"/> <i>M. fortuitum complex</i> <input type="checkbox"/> Rapid Grower <input type="checkbox"/> Other Mycobacterium																																																							
BY: <input checked="" type="checkbox"/> ZIEHL-NEELEN <input type="checkbox"/> FLUOROCHROME <input type="checkbox"/>		<table border="1"> <thead> <tr> <th rowspan="2">Drug (µg/ml)</th> <th colspan="2">Growth* at Dil.</th> <th rowspan="2">Percent of Pop.</th> </tr> <tr> <th>10^{-1}</th> <th>10^{-3}</th> </tr> </thead> <tbody> <tr> <td>CONTROL 0.0</td> <td>4+</td> <td>41c</td> <td>100</td> </tr> <tr> <td>INH 0.2</td> <td>0</td> <td>0</td> <td>0</td> </tr> <tr> <td>INH 1.0</td> <td></td> <td></td> <td></td> </tr> <tr> <td>SM 2.0</td> <td>0</td> <td>0</td> <td>0</td> </tr> <tr> <td>SM 10.0</td> <td></td> <td></td> <td></td> </tr> <tr> <td>PAS 2.0</td> <td>3+</td> <td>17c</td> <td>41</td> </tr> <tr> <td>PAS 10.0</td> <td></td> <td></td> <td></td> </tr> <tr> <td>KM 5.0</td> <td></td> <td></td> <td></td> </tr> <tr> <td>VM 5.0</td> <td></td> <td></td> <td></td> </tr> <tr> <td>THA 5.0</td> <td></td> <td></td> <td></td> </tr> <tr> <td>CS 20.0</td> <td></td> <td></td> <td></td> </tr> <tr> <td>ETH 5.0</td> <td></td> <td></td> <td></td> </tr> </tbody> </table>			Drug (µg/ml)	Growth* at Dil.		Percent of Pop.	10^{-1}	10^{-3}	CONTROL 0.0	4+	41c	100	INH 0.2	0	0	0	INH 1.0				SM 2.0	0	0	0	SM 10.0				PAS 2.0	3+	17c	41	PAS 10.0				KM 5.0				VM 5.0				THA 5.0				CS 20.0				ETH 5.0				<input type="checkbox"/> Found <input type="checkbox"/> Not Found <input type="checkbox"/> Rapid <input checked="" type="checkbox"/> Few <input type="checkbox"/> Numerous FUNGI OR YEAST: <input type="checkbox"/> Found <input type="checkbox"/> Not Found <input type="checkbox"/> Split Given to Mycology Lab.	
Drug (µg/ml)	Growth* at Dil.		Percent of Pop.																																																									
	10^{-1}	10^{-3}																																																										
CONTROL 0.0	4+	41c	100																																																									
INH 0.2	0	0	0																																																									
INH 1.0																																																												
SM 2.0	0	0	0																																																									
SM 10.0																																																												
PAS 2.0	3+	17c	41																																																									
PAS 10.0																																																												
KM 5.0																																																												
VM 5.0																																																												
THA 5.0																																																												
CS 20.0																																																												
ETH 5.0																																																												
ACID FAST BACILLI: <input checked="" type="checkbox"/> Found <input type="checkbox"/> Not Found <input type="checkbox"/> Rapid <input checked="" type="checkbox"/> Few <input type="checkbox"/> Numerous FUNGI OR YEAST: <input type="checkbox"/> Found <input type="checkbox"/> Not Found <input type="checkbox"/> Split Given to Mycology Lab.		*4 + = > 500, 3 + = 200 - 500, 2 + = 100 - 200, 1 + = 50 - 100 <50 recorded & followed by "c", as 10C = 10 colonies			<input type="checkbox"/> <i>M. scrofulaceum</i> <input type="checkbox"/> <i>M. szulgai</i> <input type="checkbox"/> <i>M. goodii</i> <input type="checkbox"/> <i>M. flourescens</i> <input type="checkbox"/> <i>M. avium complex (intracellulare)</i> <input type="checkbox"/> <i>M. xenopi</i> <input type="checkbox"/> <i>M. terrae complex</i> <input type="checkbox"/> <i>M. triviale</i> <input type="checkbox"/> <i>M. gastri</i> <input type="checkbox"/> <i>M. fortuitum complex</i> <input type="checkbox"/> Rapid Grower <input type="checkbox"/> Other Mycobacterium																																																							
FINAL REPORT MAY REQUIRE 8 WEEKS		TENTATIVE IDENTIFICATION:			<input type="checkbox"/> <i>M. xenopi</i> <input type="checkbox"/> <i>M. terrae complex</i> <input type="checkbox"/> <i>M. triviale</i> <input type="checkbox"/> <i>M. gastri</i> <input type="checkbox"/> <i>M. fortuitum complex</i> <input type="checkbox"/> Rapid Grower <input type="checkbox"/> Other Mycobacterium																																																							
SPECIMEN UNSATISFACTORY FOR TESTING BECAUSE: <input type="checkbox"/> Insufficient Amount <input type="checkbox"/> Leaked in Transit <input type="checkbox"/> Not mycobacterium <input type="checkbox"/> Contaminated <input type="checkbox"/>																																																												
Date Received and Laboratory Specimen Number Month Day Year Form No. Specimen Number		Date Reported 1st 2nd 3rd		Form Control Number																																																								

SAMPLE LABORATORY REPORT FORM

Figure 45. Drug susceptibility test. From Vestal (10).

**VI. QUALITY CONTROL
PROCEDURES FOR THE LEVEL II
MYCOBACTERIOLOGY LABORATORY**



VI. QUALITY CONTROL PROCEDURES FOR THE LEVEL II MYCOBACTERIOLOGY LABORATORY

A. INTRODUCTION

These recommendations are intended to strengthen the quality control procedures in microbiology laboratories qualified to handle mycobacterial cultures. They have been used with success in laboratories at the Center for Disease Control (CDC). Although the recommendations are not intended for small laboratories that provide only limited services, they (or some appropriate modifications) will insure the reliability of test results even in the smaller clinical laboratories.

For a quality control program to be of value, it must be workable and practical; otherwise, it will not serve the intended purpose. Quality control is the responsibility of all laboratory personnel; however, if a laboratory is able to assign at least one person to quality control, this individual should be well qualified in microbiology.

When applicable, quality control procedures must meet the requirements of the Clinical Laboratories Improvement Act of 1967.

The keys to the development of a workable quality control program are (1) adequately trained, interested, and committed personnel; and (2) common sense use of practical procedures.

B. GENERAL RECOMMENDATIONS

1. All quality control records should be retained in files or note books for at least 2 years.
2. Procedural manual(s) should be available for every routine procedure performed in the laboratory. The microbiologist in charge should date and initial all changes in techniques.
3. All containers of media, stains, and reagents should show the date received and the date first opened. Each material should be periodically rechecked, and if it is found to be unsatisfactory, it should be removed immediately from the laboratory. Purchases should be limited to 6 months' supplies.
4. Laboratory procedures used routinely should be those that have been published in reputable microbiological books, manuals, or journals. Procedures that have not been described in publications may be used, provided the necessary experimental control studies have been performed in a competent manner and a description of the procedure is expected to be published.

5. Laboratories should maintain the number and variety of cultures needed to check the quality of tests performed.

C. LABORATORY ARRANGEMENT AND PERSONNEL

1. Supplies, equipment, and work area should be arranged to facilitate an efficient work flow. Work areas should be kept free of dust. Benches should be swabbed at least once a day with an acceptable disinfectant.
2. Personnel employed in the tuberculosis laboratory should be tuberculin tested and X-rayed, as suggested in I.,B., pp. 6-7.

D. LABORATORY EQUIPMENT

Each item of equipment should meet the manufacturer's claims and specifications in the user's own laboratory. The user should consistently monitor equipment to assure the constant accuracy and precision necessary for quality laboratory performance.

Table 6 indicates the monitoring necessary for equipment found in the mycobacteriology laboratory.

E. GLASSWARE

1. Chipped or etched glassware should be discarded. Glassware should be free of detergents.
2. Sterilized glassware should be checked for sterility.
3. Sterile glassware should not be stored more than 3 weeks before being used.
4. Paper should not be used to cap glassware before it is sterilized; oil-free aluminum foil is recommended.

F. MEDIA, REAGENTS, AND BIOCHEMICAL TESTS

Table 7 indicates the minimum number of tests for the quality control of media, reagents, and biochemical tests. These control tests should be performed each time fresh media or reagents are made, and each time tests are run, except as otherwise noted.

"Sterile system" tests as negative controls are tests made with uninoculated medium or substrate at the same time and in the same manner, including periods of incubation, as the regular tests.

Table 6. Monitoring of Equipment

ITEM	MONITOR PROCEDURE
Autoclave	<ul style="list-style-type: none"> a. Recording thermometer advisable. Temperature recorded each run; record held in files. b. Cultural device (spore strips, spore suspension) used monthly. More frequently, if there is evidence of contamination. c. One hour established as routine time for decontamination of waste materials.
Biological Safety Cabinet (BSC)	<ul style="list-style-type: none"> a. Airflow checks: <ul style="list-style-type: none"> 1. Anemometer used to measure rate of airflow across front opening; should be 75 linear feet/minute for Class I BSC. 2. Gauge installed in exhaust duct beyond bacterial filter and marked with limits reached when airflow across front opening is 75 linear feet/minute. 3. Smoke sticks used to determine pattern of air currents. Used periodically, especially when new equipment is placed in the BSC work space, or any change is made in the room ventilation pattern. b. Filters: <p>Replaced when gauge in exhaust duct indicates that airflow across front opening has dropped below 75 linear feet/minute. CAUTION: Entire BSC must be decontaminated before filters are removed.</p> c. Germicidal ultraviolet (UV) lights: <p>UV light meter used to measure light intensity after initial burn of 100 hours. Test every 6 months. Replace lamp when reading drops to 70% of the initial reading. CAUTION: Clean UV lamps every 2 weeks with alcohol-soaked cotton. Do not purchase UV lamps far in advance of need.</p>
Centrifuge	<ul style="list-style-type: none"> a. Use tachometer. <p>Use appropriate rpm on an 8-place horizontal head or 12-place horizontal or angular head centrifuge to attain centrifugal force of 2,000 to 3,000 x g for sedimentation of mycobacteria. CAUTION: A 16-place horizontal head usually cannot achieve this centrifugal force.</p> b. Check brushes and bearings every 6 months.
Incubator, 35°C	<ul style="list-style-type: none"> a. Recording thermometer advisable. If not available, record daily reading, preferably a.m. Test temperature at several sites within incubator; thermometer should be placed in water. b. Control light by covering glass-front incubator door and restricting use of any lights inside the incubator.
CO ₂	<p>Check CO₂ content daily with CO₂ indicator, such as Fyrite.</p>
Microscope	<p>Always clean oil immersion objective after examining a positive acid-fast smear. Clean microscope after each use. Keep microscope under dust cover when not in use.</p>

Table 6. (Continued)

ITEM	MONITOR PROCEDURE
pH meter	a. Compensate for temperature with each run. b. Date buffer solutions and discard when unsatisfactory. c. Standardize with pH 4.0 and 7.0 buffers before each test or series of tests.
Water baths	Check temperature before and during use. Clean monthly.
Refrigerator 2° to 8°C	Check temperature daily. Walk-in should have recording thermometer. Connect walk-in to alarm system. Clean monthly. Defrost or check refrigerator and freezer compartment every 3 months.
Freezers 0° to - 70°C	Check daily. Connect to alarm system. Clean every 6 months.

Table 7. Quality control procedures for media, reagents, and biochemical tests

ITEM	CONTROLS	
	Positive	Negative
Catalase test, pH7-68°C CAUTION: Use of pH meter and thermometer is critical.	<i>M. fortuitum</i>	<i>M. gastri</i> and sterile system
Culture purity	Acid-fast stain and/or inoculation of plated media for isolated colonies at each transfer or subculture.	
Digestion-decontamination	Control by record of rate of culture contamination. Expected average is 3% to 5%. If less than 1%, the decontamination may be too harsh. If greater than 5% to 7%, the decontamination is too weak or the digestion is incomplete.	
Distilled water	Test for presence of acid-fast bacilli by strenuous sedimentation of about 300-ml sample, stain with acid-fast or fluorochrome stain, or use the Candida method to facilitate sedimentation of acid-fast bacilli (see Ref. 1).	
Light exposure test	<i>M. kansasii</i> Test exposure site and lamp. Repeat test when any change is made.	Negative built into test with unexposed part of culture.
Media for drug susceptibility testing	<i>M. tuberculosis</i> , known susceptible strain. Test each batch.	Drug-free medium inoculated with test culture.

Table 7. (Continued)

ITEM	CONTROLS	
	Positive	Negative
Media for isolation	Sample of each batch, incubated at least 48 hours for sterility. Check pH, color, consistency. Test ability to support growth in 2-3 weeks of small inoculum of freshly isolated drug-susceptible <i>M. tuberculosis</i> , or use H37Ra strain of <i>M. tuberculosis</i> .	
Niacin test, standard or strip test.	<i>M. tuberculosis</i>	Sterile system; <i>M. smegmatis</i>
Nitrate reduction, standard or strip test.	<i>M. kansasii</i> (not for strip) <i>M. tuberculosis</i> , or sterile system with powdered zinc	Sterile system without zinc
Stains	<i>M. tuberculosis</i> , mature but not old	CAUTION: Use CLEAN, NEW slides. Use AFB-free water.

Additional tests may be included in the quality control program. For instance, drug-resistant strains with known MIC's may be included in the controls for drug susceptibility tests. Personnel in each laboratory must decide how many tests they will make. Whatever tests are elected as controls should be made consistently, and the results depended upon for control of the actual diagnostic procedures.

See Ellis (2) for more detailed information on quality control procedures.

G. PROCEDURE FOR THE STORAGE OF MYCOBACTERIAL STOCK CULTURES (3)

1. Place 2 ml of tap water in a 2-dram, flat-bottom, screwcap vial.
2. Cap the vials loosely and autoclave at 121°C for 15 minutes.
3. Prepare suspensions of the cultures by emulsifying five or six colonies in the 2 ml of water. Alternatively, the cultures may be grown in 7H-9 broth and bottled in 1 to 2 ml amounts in the above vials or in vaccine-stoppered vials (4,5).
4. Tightly cap the vials and store them in an upright position in a freezer at a temperature ranging from -16° to -70°C. Some species (especially *M. tuberculosis*) lose viability on prolonged storage (1 year or more) at -20°C, but all species retain virtually 100% viability when stored at -70°C.

NOTE: The contents of vials can be thawed and refrozen several times.

H. SOME SOURCES OF MYCOBACTERIAL STOCK CULTURES

1. American Type Culture Collection (ATCC).
12301 Parklawn Dr.
Rockville, Maryland 20852
2. The Curator
Mycobacterial Culture Collection
National Jewish Hospital and Research Center
3800 East Colfax Avenue
Denver, Colorado 80206

REFERENCES

1. Dizon, D., C. Mihailescu, and H. C. Bae. 1976. Simple procedure for detection of *Mycobacterium gordonae* in water causing false-positive acid-fast smears. *J. Clin. Microbiol.* **3**:211.
2. Ellis, R. J. 1974. Manual of quality control procedures for microbiological laboratories. DHEW, PHS, Center for Disease Control, Atlanta, Ga. 30333.
3. Jones, W. D., Jr. 1957. Simple method for maintaining stock cultures of *Mycobacterium* species. *Amer. J. Clin. Pathol.* **27**: 363-364.
4. Kim, T. H. and G. P. Kubica. 1972. Long term preservation and storage of mycobacteria. *Appl. Microbiol.* **24**: 311-317.
5. Kim, T. H. and G. P. Kubica. 1973. Preservation of mycobacteria: 100% viability of suspensions stored at -70C. *Appl. Microbiol.* **25**: 956-960.

VII. RECORDS AND REPORTS

VII. RECORDS AND REPORTS

If laboratory findings are to be useful, they must be communicated to the proper authorities. Clinicians use the findings in the diagnosis and management of disease; public health authorities use the findings for statistical and epidemiologic purposes.

Laboratory procedures for mycobacteriology are notoriously time-consuming, often taking weeks or even months to complete. For this reason, interim reports should be issued. They may be sent out after (1) acid-fast smears have been examined, (2) growth has been observed on the culture medium and the species has been tentatively identified or results of a direct drug susceptibility test are known, and finally, (3) the species has been precisely identified on the basis of detailed in vitro tests and/or indirect drug susceptibility tests have been completed.

Obviously, all of the technical details of each laboratory test cannot be recorded on the reports, but they should be precisely outlined in the laboratory procedural manual, and the results must be a part of the daily laboratory records. The validity of these results also must be insured by including appropriate quality controls with each batch of media or each test run.

The interim reports may be provided to the clinician by mailing copies of the original report form as new information becomes available, or by using precarboned, sectioned, perforated forms. In the latter case, the appropriate section of the form may be completed, dated, detached, and mailed. The final report will have all of the data previously reported, so earlier interim reports can be destroyed and only the final report retained in the patient's file.

Thus, smear reports should be completed and returned to the clinician within a few days of receipt of specimen; contaminated cultures may be noted within the first 7-10 days and repeat specimens requested; preliminary (tentative) identification and/or direct drug susceptibility test results should be available within 3-4 weeks; all test results may not be available for 6 to 9 weeks.

A sample report form is shown on the next page.

Sample Report Form

LABORATORY NAME & ADDRESS			FORM NAME & NUMBER		
Patient Name _____				Age _____	Sex _____
Address _____					
City _____		State _____		Zip Code _____	
Social Security Number _____					
Please Return Report To _____		Physician's Code _____		To be Filled in by Physician	
Dr. _____		Mo. _____ Day _____ Year _____		Date Specimen Collected _____	
_____		SPECIMEN:		Patient on Therapy? <input type="checkbox"/> Yes <input type="checkbox"/> No	
_____		<input type="checkbox"/> Natural		<input type="checkbox"/> Mx <input type="checkbox"/> Culture <input type="checkbox"/> Drugs	
_____		<input type="checkbox"/> Induced		<input type="checkbox"/> INH <input type="checkbox"/> PAS	
_____		<input type="checkbox"/> Gastric		<input type="checkbox"/> SM <input type="checkbox"/> ETA	
_____		<input type="checkbox"/> Urine		<input type="checkbox"/> EMB <input type="checkbox"/> KM	
_____		<input type="checkbox"/> Other		<input type="checkbox"/> RIF <input type="checkbox"/> _____	
(Specify) _____		<input type="checkbox"/> Culture		If repeat, date of last specimen: Mo. _____ Day _____ Year _____	
INTERIM REPORT #1		ACID FAST BACILLI:		Fungi or Yeast	
EXAMINED MICROSCOPICALLY		<input type="checkbox"/> Found <input type="checkbox"/> Not found		<input type="checkbox"/> Found <input type="checkbox"/> Not found	
By <input type="checkbox"/> Ziehl-Neelsen		<input type="checkbox"/> 1-2 per 300 fields		<input type="checkbox"/> Split sent to Mycology	
<input type="checkbox"/> Fluorochrome		<input type="checkbox"/> 1-9/100 fields <input type="checkbox"/> 1-9/10 fields		<input type="checkbox"/> Found <input type="checkbox"/> Not found	
<input type="checkbox"/> _____		<input type="checkbox"/> 1-9/field <input type="checkbox"/> >9/field		<input type="checkbox"/> Split to Gen'l. Bact'y.	
				SPECIMEN UNSATISFACTORY FOR TESTING BECAUSE:	
				<input type="checkbox"/> Insufficient Amount	
				<input type="checkbox"/> Leaked in transit	
				<input type="checkbox"/> Contaminated (Send Another)	
INTERIM REPORT #2			REPORT #3		
DRUG SUSCEPTIBILITY TESTS IN 7H-10 AGAR			<input type="checkbox"/> FINAL REPORT <input type="checkbox"/> Tentative Report		
<input type="checkbox"/> Direct <input type="checkbox"/> Indirect			<input type="checkbox"/> Isolate specifically identified as <i>Mycobacterium tuberculosis</i>		
Drug	µg/ml	Growth* at dil'n	<input type="checkbox"/> Culture sent to Reference Laboratory Final report may take 4-6 weeks.		
CONTROL			Tentative identification or rough grouping suggests		
INH	0.2		<input type="checkbox"/> <i>M. tuberculosis</i>		
INH	1.0		<input type="checkbox"/> Photochromogen <input type="checkbox"/> Scotochromogen		
SM	2.0		<input type="checkbox"/> Nonphotochromogen <input type="checkbox"/> Rapid Grower		
SM	10.0				
RIF	1.0				
RIF	5.0				
EMB	5.0				
PAS	2.0				
ETA	5.0				
KM	5.0				
*4+ = >500, 3+ = 200-500, 2+ = 100-200, 1+ = 50 colonies, <50 recorded, followed by "c", as 10c = 10 colonies.			Date reports mailed: 1st _____ 2nd _____ 3rd _____		
If >1% of tested population exhibits resistance to given drug, this suggests emergence of drug resistance.			Date specimen rec'd. _____ Lab. specimen No. _____		

(For Training Use Only)



VIII. APPENDIX

APPENDIX

A. PREPARATION AND STORAGE OF MEDIA

1. Preparation of Media

Perhaps more important than the kind of medium selected for the culture of mycobacteria is the care employed in its preparation. All glassware and equipment should be thoroughly cleaned and sterile (when so indicated). To insure reproducibility of media, all reagents must be freshly prepared from chemicals of certified purity and freshly distilled water. Media formulations and directions for preparation should be precisely followed. The media preparation room should be clean, free of dust, and preferably in an isolated area where traffic flow is minimal; wet mopping of floors, swabbing counters with disinfectant, dust-free filters in air intakes, and ultraviolet (UV)-irradiation of upper air (when room is not in use) all serve to minimize air contaminants. Strict aseptic technique should be used when adding heat-labile enrichments or drugs to media. Sterilizing capabilities of filters (Seitz, sintered glass, or membrane) and autoclaves must be monitored periodically to insure that they are functioning properly. Excessive light and heat must be avoided in the preparation of the various types of Middlebrook media (7H-9, 7H-10, 7H-11), to minimize chances of toxic formaldehyde forming (5). Adding 0.1% L-aspartic acid (potassium salt) (3) to the basal 7H-10 medium greatly enhances niacin production by *M. tuberculosis* growing on this clear agar medium, but aspartate also interferes with the action of certain drugs that might be used for susceptibility tests, so the microbiologist must be aware of this disadvantage (3).

a. Modified Lowenstein-Jensen Egg Medium (2).

Fresh eggs, not more than 1 week old, are cleaned by scrubbing with a hand brush in a soap solution for 30 minutes. Rinse thoroughly in running water, then soak eggs in 70% alcohol for 15 minutes. With well-scrubbed hands, break the eggs into a sterile flask and homogenize by hand shaking. Filter eggs through four layers of sterile gauze into a sterile, graduated cylinder.

- (1) For salt solution combine:

Monopotassium phosphate (anhydrous)	2.4g
Magnesium sulfate. 7H ₂ O.	0.24g
Magnesium citrate	0.6g
Asparagine	3.6g
Glycerol (reagent grade).	12.0ml
Distilled water.	600.0ml
- (2) Add potato flour. 30.0g
- (3) Autoclave at 121° C for 30 minutes
Cool to room temperature
- (4) Add malachite green (2% aqueous solution
freshly prepared). 20.0ml
- (5) Add homogenized whole eggs 1000.0ml
- (6) Mix and pour into a sterile aspirator bottle or fun-
nel with a bell attachment (test tube filling device)
and dispense.
- (7) Place approximately 6 to 8 ml medium into each
20 x 150-mm sterile screwcap test tube.
- (8) Slant tubes and coagulate by inspissation at 85°C
for 50 minutes
- (9) Incubate at 37°C for 48 hours as a sterility check.
- (10) Medium may be stored in the refrigerator for sev-
eral months if caps are tightly closed to prevent
evaporation.

b. **Preparation of 7H-10 Agar from Commerical Base (4).**

Note: Experience has shown that boiling the basal medium before autoclaving produces a medium of inferior quality. This result may be avoided by preparing the medium in 200 ml amounts and autoclaving for 10 minutes.

To prepare 200 ml complete medium, follow these directions precisely:

- (1) Suspend 3.6 g of Middlebrook 7H-10 basal medium in 180 ml of freshly distilled water and add 1.0 ml of reagent grade glycerol.
- (2) Swirl base into suspension and sterilize in the autoclave for 10 minutes at 121°C.
- (3) Remove medium from autoclave as soon as the pressure will allow and place in a water bath at 50° to 56°C.

- (4) As soon as cooled to 50° to 56°C, add 20 ml of OADC enrichment.
- (5) For drug media, cool to 50° to 52°C and then add the required amount of drug.
- (6) Dispense within 1 hour after autoclaving.
- (7) Allow to solidify at room temperature without exposure to daylight (fluorescent light is all right).

NOTE: When the autoclaved medium is allowed to stand for more than 1 hour in the water bath, a precipitate begins to form. Experience has shown that this practice should be avoided. Pour as soon as possible. Always check the medium when taking it out of the autoclave. It should be clear and transparent. If a precipitate is present, it may be due to (a) sterilization temperature within the autoclave being reached too rapidly to dissolve the base in 10 minutes, (b) poor quality basal medium, and/or (c) dirty glassware.

CAUTION: Never autoclave 7H-10 medium base and store it in the refrigerator for future use. The heat required to re-melt it produces a medium of poor quality.

For best results do not prepare in lots of more than 400 ml.

c. **Preparation of 7H-11 Agar from Commercial Base (1).**

Seven H-11 (7H-11) agar is a modification of Middlebrook 7H-10 agar and was formulated by Cohn, Waggoner, and McClatchy (1), who demonstrated that adding an enzymatic digest of casein to 7H-10 medium stimulated the growth of the more fastidious strains of *M. tuberculosis*.

To prepare 200 ml complete medium:

- (1) Suspend 3.78 g of 7H-11 agar basal medium in 180 ml distilled water containing 1 ml glycerol.
- (2) Autoclave for 15 minutes at 121°C.
- (3) Cool to 50° to 52° C, add 20 ml OADC enrichment.
- (4) For drug media, cool to 50° to 52° C, then add drugs.

(5) Dispense within 1 hour after autoclaving.

NOTE: Observe precautions described in preparation of 7H-10 medium.

d. Preparation of 7H-9 Broth from Commercial Base (4).

To prepare 900 ml complete medium:

- (1) Suspend 4.7 g of the dehydrated medium in 900 ml distilled water containing 2 ml glycerol, or 0.5 g (ml) Tween 80. Do not use Tween 80 and glycerol together.
- (2) Autoclave at 121° C for 15 minutes.
- (3) Remove from autoclave and cool to 45°C.
- (4) As soon as cooled to 45°C, aseptically add 100 ml of ADC enrichment.
- (5) Aseptically dispense 5-ml amount into 20-x 150-mm screwcap test tubes.

2. Storage of Media

- a. Always protect any media from direct light.
- b. Place plates in clean plastic bags or sterile dry containers.
- c. Tighten caps of screwcap tubes before storing.
- d. Media kept for longer than 1 week should be kept in the refrigerator to prevent dehydration.
- e. Media showing signs of contamination and dehydration should be discarded.

B. NITRATE REDUCTION STANDARDS

STOCK SOLUTIONS

1. 0.067 M disodium phosphate
2. 0.067 M monopotassium phosphate
3. 0.067 M trisodium phosphate
4. 1% phenolphthalein (1 gram in 100 ml 95% ethyl alcohol)
5. 1% brom thymol blue (1 gram in 100 ml 95% ethyl alcohol)
- 5A. 0.01% brom thymol blue: prepared by mixing 1.0 ml of stock No.5 above in 100 ml distilled water.

WORKING BUFFER SOLUTION

Mix:

Stock No. 1	35 ml
Stock No. 2	5 ml
Stock No. 3	100 ml

PREPARATION OF STANDARDS

1. Line up eight clean test tubes in a rack. Use tubes the same size as for the test.
2. Put 2 ml of working buffer solution into seven tubes.
3. To 10 ml of working buffer solution, add 0.1 ml of stock No. 4 and 0.2 ml of stock No. 5A.
4. Add 2 ml of the step 3 solution to the 8th tube. This is the 5+ color standard.
5. To the first tube in the series of seven tubes, add 2 ml of the step 3 solution. Mix well and carry over 2 ml to the next tube. Continue to make serial dilutions, discarding 2 ml from the 7th tube.

6. The color standard:

tube in step 4 =	5+
tube 1 =	4+
tube 2 =	3+
tube 4 =	2+
tube 5 =	1+
tube 7 =	±

These colors should range from pink (\pm) to purplish red (5+) (see Fig. 46).

7. Autoclave tubes, seal, and store in a refrigerator.

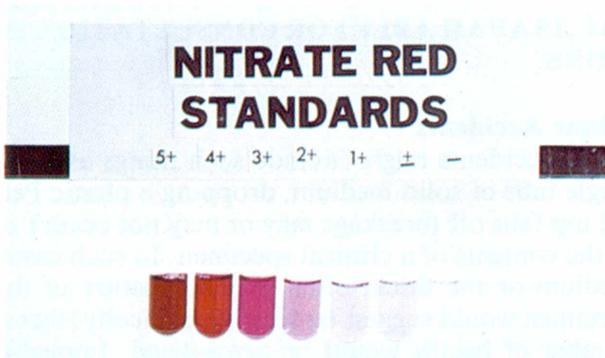


Figure 46. Nitrate reduction standards.

C. IN CASE OF ACCIDENT

“Laboratory Safety” doesn’t just happen. It is the end result of: (1) the recognition that accidents can, will, and usually do occur at the most unpropitious times; (2) an open, rational discussion of ways to minimize or prevent such accidents; and (3) the formulation of an effective plan to neutralize as quickly as possible the potentially harmful effects of those accidents which do occur.

In the Mycobacteriology Laboratory we all hope that a laboratory accident never occurs, but we must anticipate the eventuality and have a well-formulated plan to minimize the spread of potentially infectious mycobacteria. No laboratory accident should be regarded lightly, yet not all will be handled in the same manner; there will be room for personal judgment. Personnel should be instructed and urged to think about and discuss the possible accidents—the things that could “go wrong”—associated with each laboratory procedure they use.

Perhaps the most feared accidents are those involving the release of tubercle bacilli or other mycobacteria into the laboratory air. The propensity of such airborne bacilli to be carried to and to infect personnel at locations distant from the site of the accident (especially in buildings having a recirculating air handling system) is great.

The suggestions below are made only on the presumption that your institution has a one pass (non-recirculating) air handling system which is recommended for those laboratories engaged in work with mycobacteria.

NOTE: IF YOU WORK IN A LABORATORY WITH A RECIRCULATING AIR HANDLING SYSTEM, YOUR PROBLEMS OF CONTAGION CONTAINMENT ARE GREATLY MAGNIFIED, AND IT IS NECESSARY TO INDIVIDUALIZE YOUR PLANS TO PROTECT PERSONNEL FROM INFECTION. THE LABORATORY TRAINING AND CONSULTATION DIVISION, CDC, IS AVAILABLE FOR CONSULTATION IN THESE SITUATIONS.

1. Minor Accidents

These accidents might include such things as breakage of a single tube of solid medium, dropping a plastic Petri dish so the top falls off (breakage may or may not occur), or spillage of the contents of a clinical specimen. In such cases the solid medium or the thick, often mucoid, nature of the clinical specimen would suggest (at least theoretically) that a minimal number of bacilli would be aerosolized. Immediate action should be taken to cover the spill to minimize further aero-

solization (cover the site with a large cloth or your lab coat). Soak the cloth with disinfectant (e.g., 3% Amphyl) to wet the area, thereby minimizing further aerosol formation. Exit the room and stay out for 2 to 4 hours to enable the air handling system to evacuate most of the aerosolized bacilli. When you re-enter, wear protective clothing (a supply should be available outside the TB room) and mask, carefully clean up the spill (autoclave culture tubes, plates, and cloths), and mop the floors and countertops with disinfectant. Note that in anticipation of such accidents there should be available a supply of large cloths and a reservoir of disinfectant which is readily accessible and can be poured quickly and easily.

2. Major Accidents

A major accident cannot be categorized exclusively by the volume or quantity of the breakage or spill, even though this is one measuring parameter. More important is whether the material involved is a liquid and, if so, what is the concentration of mycobacteria in the suspension. Thus, one or two tubes, or a flask of liquid culture containing 10^8 per ml could generate a cloud of droplet nuclei capable of infecting large numbers of people exposed to it. Here is where "personal judgment" will play a big role. Appropriate action would be:

- a. Evacuate the room immediately. The danger from the potentially infectious aerosol is greater than any need to "cover the spill."
- b. Leave the BSC operating and do not re-enter the room for at least 4 hours. This will enable considerable dilution of the infectious droplet nuclei. Moreover, evacuation of the air through the HEPA filter system of the BSC should reduce the likelihood of people outside the building becoming infected.
- c. Decontaminate the room(s) after the 4-hour waiting period by using formaldehyde gas. Because of the ready availability of 36% to 40% formalin in most laboratories, this procedure will be outlined:
 - (1) Seal all air intake and exhaust grills in the room. This may be as simple as taping large plastic garbage bags over the grills, or as sophisticated as having a flanged frame over each grill which will accept a metal or solid plastic sheet that can be taped in place to insure tightness. In similar fashion, tape around door frames or other openings through which the formaldehyde may leak.

- (2) Use an electric hot plate to boil off 1 ml of formalin per cu. ft. of room space. Example: a room 10x12x10 feet would have 1200 cu. ft. of space and would be treated with 1200 ml of formalin.

CAUTION: DO NOT OVERDOSE THE ROOM WITH FORMALDEHYDE, BECAUSE EXPLOSIVE CONCENTRATIONS CAN BE ATTAINED. THE AMOUNTS LISTED HERE ARE WELL WITHIN THE SAFETY RANGE.

- (3) Raise the relative humidity of the room to about 70% to insure optimal effect of the formaldehyde. Most Chemistry or Physics handbooks have tables of the amount of water that can be held in a given volume of air at full saturation; you need only 70% of this amount. Calculate the quantity needed and boil it off on an electric hot plate. Example: In our 1200 cu. ft. room; the amount of water needed to fully saturate the room at 70°F (21°C) is 18.45 g (or ml) per cubic meter (or per 35.3 cu. ft.). By simple mathematics, 1200 cu. ft. divided by 35.3 cu. ft. in 1 cubic meter x 18.45 g/cubic meter x 0.7 (for 70% humidity) equals 439 g (or ml) of water. If 500 ml of water is boiled off in the room at the same time (or just before) the formaldehyde is vaporized, the desired humidity should be attained.
- (4) Allow the formaldehyde to stay in the room overnight. Put on a gas mask* to enter the room and remove the covers from air intake and exhaust grills. Allow the room to air until no more formaldehyde is detectable, then mop all residue from the floors, walls, and counters. If a white, powdery residue is obvious, this may be removed with 10% ammonium hydroxide (use gloves).

*MSA Chin Type Gas Mask with Ultravue Facepiece available from Mine Safety Appliances Co., 400 Penn Center Blvd., Pittsburgh, PA 15235. Should be used with Chin Type Canister, Type GMR. Approximate cost with canister is \$90.00 (1979-80).

REFERENCES

1. Cohn, M. L., R. F. Waggoner, and J. K. McClatchy. 1968. The 7H-11 medium for the cultivation of mycobacteria. *Amer. Rev. Respir. Dis.* **98**:295-296.
2. Holm, J. and V. Lester. 1941. Diagnostic demonstration of tubercle bacilli. *Acta. Tuberc. Scand.* *16* :Fasc. 3-4. *In Pub. Hlth. Rep.* **62**:847-854, 1947.
3. Kilburn, J. O., K. D. Stottmeier, and G. P. Kubica. 1968. Aspartic acid as a precursor for niacin synthesis by tubercle bacilli grown on 7H-10 agar medium. *Amer. J. Clin. Pathol.* **50**:582-586.
4. Middlebrook, G., and M. L. Cohn. 1958. Bacteriology of tuberculosis: laboratory methods. *Amer. J. Pub. Hlth.* **48**:844-853.
5. Miliner, R. A., K. D. Stottmeier, and G. P. Kubica. 1968. Formaldehyde: A photothermal activated toxic substance produced in Middlebrook 7H-10 medium. *Amer. Rev. Respir. Dis.* **99**:603-607.

